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ESTRATÉGIAS DE CONTROLE E DESCONTAMINAÇÃO DO TRIGO EM GRÃOS (*Triticum aestivum* L.) COM RELAÇÃO A FUNGOS, MICOTOXINAS E AGROTÓXICOS UTILIZANDO COMPOSTOS QUÍMICOS E OZÔNIO GASOSO

Tese apresentada ao Programa de Pós-graduação em Ciências dos Alimentos do Centro de Ciências Agrárias da Universidade Federal de Santa Catarina, como requisito final à obtenção do título de Doutor em Ciência dos Alimentos.
Orientadora: Dra. Vildes Maria Scussel

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GEOVANA DAGOSTIM SAVI BORTOLOTTO

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RESUMO

O trigo em grãos (*Triticum aestivum* L.) é uma cultura altamente consumida pela população diariamente, sendo matéria prima para produção de farinhas, massas, produtos de panificação e culinária em geral. As regiões produtoras de trigo em grãos no Brasil e no mundo se concentram principalmente nas zonas de clima temperado, onde o excesso de chuvas, aliado à temperaturas elevadas, favorecem o aparecimento de contaminantes no trigo, tais como insetos, fungos e micotoxinas. Para o controle destes contaminantes, a aplicação de agrotóxicos pode ser realizada tanto no campo quanto durante o armazenamento dos grãos, no entanto, se isto ocorrer de maneira inapropriada, também pode tornar-se um problema de contaminação, quando levado em consideração a persistência dos resíduos de agrotóxicos neste alimento. Além disso, muitos destes contaminantes são resistentes ao processo de moagem e aquecimento no processamento e permanecem nos subprodutos do trigo, e por isso, podem entrar na cadeia alimentar de animais e humanos diretamente. Diante deste contexto, o objetivo foi estudar estratégias de controle e descontaminação do trigo (*Triticum aestivum* L.) em grãos com relação à fungos, micotoxinas e resíduos de agrotóxicos, utilizando compostos químicos e ozônio gasoso. Os grãos de trigo integral utilizados na pesquisa apresentaram importantes espécies fúngicas (*Fusarium graminearum*, *Fusarium verticillioides*, *Penicillium citrinum*, *Aspergillus flavus* e *Aspergillus parasiticus*), sendo que 47,2% das amostras apresentaram contaminação por deoxinivalenol (DON), principal toxina produzida por *F. graminearum* e uma das mais encontradas em grãos de trigo mundialmente. Com relação a descontaminação por compostos químicos, estes foram testados frente a importantes cepas fúngicas encontradas no trigo. Nos estudos *in vitro*, os melhores resultados encontrados para as nanopartículas de ouro foram com as cepas de *F. verticillioides* e *A. flavus*. Já as nanopartículas de zinco e outros compostos de zinco, como óxido, sulfato e perclorato de zinco, apresentaram resultados significativos na inibição de *F. graminearum* e *P. citrinum* e na redução das micotoxinas DON, aflatoxina (AFB_1) e citrinina (CTR), sendo que algumas foram reduzidas totalmente por alguns dos compostos testados. Em adição, alterações no metabolismo celular dos fungos foram observados na produção de conídios e formação das hifas, auxiliando na compreensão do mecanismo de ação destes compostos frente aos fungos e na formação das micotoxinas. Nos estudos *in vivo*, realizados sobre as

plantas de trigo, os compostos demonstraram eficiência no controle do crescimento de *F. graminearum* e na formação de DON durante o cultivo das plantas de trigo. Além disso, a concentração de zinco aplicada nos grãos de trigo permaneceu dentro dos níveis recomendados internacionalmente para o consumo diário de zinco e em adição, nenhuma alteração morfológica nos grãos foi observada após o tratamento. Diante dos resultados encontrados, podemos concluir que novas estratégias de controle com estes compostos, especialmente os de zinco, juntamente com o tratamento convencional e as medidas preventivas, poderiam reduzir o crescimento de fungos e formação das micotoxinas, frequentemente encontradas nos grãos de trigo.

Com relação a descontaminação por gás ozônio em estudos *in vitro*, este mostrou eficiência na redução de fungos, especialmente *F. graminearum* e *P. citrinum*, e em adição apresentaram maior sensibilidade quanto a germinação dos conídios, alterações morfológicas, mortalidade e aumento da produção de espécies reativas de oxigênio nas hifas. Nos estudos *in vivo*, realizado nos grãos de trigo, o gás ozônio mostrou ser efetivo na inibição do crescimento de *F. graminearum*, *A. flavus* e *P. citrinum* e também na degradação das micotoxinas DON, aflatoxinas (AFB_1, AFB_2, AFG_1 e AFG_2) e CTR produzidas por estes fungos. Em adição, o gás ozônio demonstrou potencial na degradação dos resíduos de agrotóxicos, especialmente os inseticidas fenitrotiona e deltametrina, utilizados frequentemente nos grãos de trigo armazenados para evitar a proliferação de insetos. Além disso, análises físico-químicas do trigo em grãos, tais como: conteúdo de carbonil e carboxil, difração de raio-X, peroxidação lipídica, análise de proteínas, análise da microestrutura e por fim a germinação das sementes não foram alteradas após o tratamento nas concentrações eficientes para a redução dos contaminantes estudados. Neste sentido, o gás ozônio que tem vantagens de ser internacionalmente reconhecido como seguro, além de não deixar resíduos nos alimentos, pode ser um método promissor de descontaminação a ser aplicado nas indústrias e unidades armazenadoras durante o período de armazenamento dos grãos de trigo, a fim de reduzir a contaminação e garantir a segurança do alimento e do consumidor.

Palavras-chaves: Fungos. Micotoxinas. Agrotóxicos. Compostos químicos. Gás ozônio. Trigo em grãos.

ABSTRACT

Wheat (*Triticum aestivum* L.) is a crop daily consumed by the population and raw material for the production of flour, pasta, bakery products and cooking in general. Wheat producing regions in Brazil and in the world are concentrated mainly in the temperate zones, where excess rainfall, together with high temperatures induce the appearance of contaminants in the wheat, such as insects, fungi and mycotoxins. To control these contaminants, pesticide application can be performed both in the field and storage grains, however, if occur inappropriately, it can also become a problem of contamination, when taken into account the persistence of food pesticide residues. Moreover, some these contaminants are resistant to milling and heating process and remain in the processing of wheat by-products, and therefore may enter the food chain of animals and humans directly. In this context, the aim of this study was to evaluate the control strategies and decontamination of wheat grain with respect to fungi, mycotoxins and pesticide residues using chemical compounds and ozone gas. The whole wheat grains used in the study showed the presence of important fungal species (*Fusarium graminearum*, *Fusarium verticillioides*, *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus*) and 47.2% of the samples were contaminated with deoxynivalenol (DON), main toxin produced by *F. graminearum* and one of the most found in wheat grains worldwide. With respect to the decontamination using chemical compounds, *in vitro* studies showed that the best results for gold nanoparticles were found to *F. verticillioides* and *A. flavus* strains. Since zinc nanoparticles and zinc compounds such as zinc oxide, sulfate and perchlorate, showed significant results to *F. graminearum* and *P. citrinum* inhibition and mycotoxins DON, aflatoxin (AFB_1) and citrinin (CTR) reduction, being that some of the tested compounds mycotoxins were completely reduced. In addition, changes in cell metabolism of fungi were observed in terms of conidia production and hyphae formation, helping to understand the action mechanism of these compounds toward fungi and mycotoxins formation. The *in vivo* studies conducted on wheat plants, showed that the compounds were efficient to inhibit *F. graminearum* growth and DON formation reduction during cultivation. Moreover, the zinc concentration applied to the wheat grains remained within the internationally recommended zinc daily intake and additionally as well morphological changes were not observed in the grains after treatment. Considering the results, we conclude that new control strategies with these compounds, especially for zinc compounds,

together with conventional treatment and preventive measures could the fungal growth and mycotoxin formation reduction, often found in wheat grains.

Regarding to ozone gas decontamination *in vitro* studies, they showed efficiency on fungi reduction, particularly to *F. graminearum* and *P. citrinum* and a higher sensitivity for conidia germination, morphological changes, mortality and increase of reactive oxygen species production in the hyphae. In studies *in vivo* conducted in the grains wheat, demonstrasted tha ozone gas was effective on *F. graminearum*, *A. flavus* and *P. citrinum* growth inhibition and also in the DON, aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and CTR degradation. In addition, ozone gas showed potential in the degradation of pesticides residues, especially fenitrothion and deltamethrin insecticides, commonly used in wheat grain stored to prevent the insects proliferation. Besides, physical and chemical analysis of wheat grain, such as carboxyl and carbonyl content, X-ray diffraction, lipid peroxidation, protein analysis, microstructural analysis and finally seed germination did not were changed after treatment at concentrations effective for the contaminants reduction. In this sense, since ozone gas is internationally recognized as safe and does not leave residues in food, could be a promising method of decontamination in industries and storage units during the wheat grain storage, in order to avoid contamination and ensure security of food and consumer.

Keywords: Fungi. Mycotoxins. Pesticides. Chemical compounds. Ozone gas. Wheat grains.

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CAPÍTULO 1

LISTA DE ABREVIATURAS

AFLs – Aflatoxinas
ANVISA - Agência Nacional de Vigilância Sanitária
AOAC - *Association of Official Analytical Chemistry*
a_w - Atividade de água
CAST - Council for Agricultural Science and Technology
CCD - Cromatografia em Camada Delgada
CEC - Commission of the European Communities
CFR - Code of Federal Regulations
CIMMYT - Centro Internacional de Mejoramiento de Maíz y Trigo
CNNPA - Comissão Nacional de Normas e Padrões para Alimentos
CONAB - Companhia Nacional de Abastecimento
CTR - Citrinina
DNA - Ácido desoxirribonucléico (do inglês, *Deoxyribonucleic acid*)
DON - Deoxinivalenol
DRX - Difração de Raio-X
EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária
EROs - Espécies reativas de oxigênio
EUA - Estados Unidos da América
FAO - *Food and Agriculture Organization*
FBs - Fumonisinas
FDA - *Food and Drug Administration*
GRAS - *Generally Recognized as Safe*
HPLC - *High Performance Liquid Chromatography*
IARC - *International Association on Research of Cancer*
LMR - Limite máximo de resíduos
LMT - Limite máximo tolerável
MAPA - Ministério da Agricultura, Pecuária e Abastecimento
MEV - Microscopia eletrônica de varredura
MET - Microscopia eletrônica de transmissão
NPs - Nanopartículas
ODS - Office of Dietary Supplements
OMS - Organização Mundial da Saúde (do inglês, *World Health Organization*)
OTA - Ocratoxina A
PARA - Programa de Análise de Resíduos de Agrotóxicos em Alimentos
PAT - Patulina
RDC - Resolução da Diretoria Colegiada
RNA - Ácido ribonucléico (do inglês, *Ribonucleic acid*)

SCF - *Scientific Committee on Food*
SNC - Sistema nervoso central
T_2 - Toxina T_2
UE - União Européia
UFC - Unidade formadora de colônia
USEPA - United States Environmental Protection Agency
ZON – Zearalenona

SUMÁRIO

1 INTRODUÇÃO

O trigo representa a segunda maior cultura de grãos em produção no mundo, sendo superado apenas pelo milho. No Brasil, a produção de trigo (*Triticum aestivum* L.) é de aproximadamente 5,09 milhões de toneladas (ton) por ano, sendo a região Sul responsável por 94% desta produção. O consumo anual de trigo no país tem se mantido em torno de 10 milhões de ton, sendo que a demanda atual de trigo importado é de aproximadamente 5,0 milhões de ton. Portanto, os principais fornecedores responsáveis por suprir a demanda de trigo no Brasil são a Argentina (84%), EUA (7%), Polônia (6%), Canadá (2%) e outros países (1%) (CONAB, 2012). Estas grandes regiões produtoras de trigo no mundo se concentram principalmente nas zonas de clima temperado, onde o excesso de chuvas, aliado à temperaturas elevadas, favorecem o aparecimento de contaminantes no trigo, tais como insetos, fungos e micotoxinas.

Entre estes, os fungos toxigênicos podem amplamente se desenvolver tanto nas plantas no campo, quanto no período de armazenamento dos grãos. *Fusarium* sp., especialmente *F. graminearum* (teleomorfo *Gibberella zeae*), pode ser facilmente encontrado como contaminante dos grãos no campo ocasionando uma doença chamada giberela do trigo. Esta doença é reportada muldialmente pelo seu difícil controle na planta, isto porque o fungo é capaz de sobreviver saprofiticamente nos restos culturais de muitas espécies cultivadas e os seus propágulos serem transportados a longas distâncias pelo vento. Além disso, a presença de grãos giberelados está associado a produção de deoxinivalenol nos grãos de trigo. Já os gêneros *Aspergillus* sp. e *Penicillium* sp. podem se desenvolver comumente durante armazenagem dos grãos em condições inadequadas (alta temperatura e umidade), e produzir aflatoxina, citrinina, ocratoxina, além de outras micotoxinas de armazenagem. Estas micotoxinas podem causar danos a várias funções do organismo humano e animal, muitas vezes levando ao desenvolvimento de tumores, podendo inclusive ser letal.

Tendo em vista a presença de micotoxinas no trigo relatados em todo mundo, os níveis de contaminação neste alimento torna-se um problema de saúde pública, uma vez que cerca de 21% dos alimentos dependem da cultura do trigo, matéria prima para produção de farinhas, massas, produtos de panificação e culinária em geral. Além disso, estas toxinas podem ser resistentes ao processo de moagem e aquecimento no processamento, e por isso, entram na cadeia alimentar de animais e humanos diretamente.

Nesse contexto, desde o ano de 2011, a Agência Nacional de Vigilância Sanitária dispõe de uma nova legislação para limites máximos toleráveis (LMT) para micotoxinas em trigo (BRASIL, 2011, 2013). O limite está sendo reduzido ao longo dos anos para permitir que os produtores de grãos possam adaptar-se a legislação sem causar escassez do alimento. Nesta nova legislação o prazo limite para adequação do LMT das micotoxinas nos alimentos é até 2017, sendo assim, o mercado produtor tem urgência na prevenção e eliminação destes contaminantes.

A fim de conhecer se a qualidade do trigo produzido no Brasil está de acordo com o esperado pela nova legislação, é necessário realizar levantamentos para investigar a presença de fungos toxigênicos e a quantificação de micotoxinas no trigo, principalmente na região Sul, a maior produtora do país. Mesmo que todas as medidas preventivas sejam adotadas constantemente no controle da contaminação do trigo, muitas vezes, estas não são suficientes para manter a qualidade do alimento. Neste sentido, a descontaminação se faz necessária, sendo essenciais os estudos de novos agentes antifúngicos que possam auxiliar nas estratégias atuais de controle.

Com base em todos os possíveis contaminantes citados, serão utilizados neste estudo métodos químicos de descontaminação, tais como: compostos inorgânicos (nanopartículas e compostos de zinco) e o tratamento com gás ozônio. As nanopartículas e compostos de zinco são vantajosos devido a forte atividade anti-microbiana em baixas concentrações. No caso do zinco, são elementos essenciais ao corpo humano, sendo autorizados para fortificação de alimentos e considerados como seguros para o uso em alimentos em quantidades adequadas. Mesmo apresentando estas vantagens, poucos são os estudos que comprovam o seu mecanismo de ação frente a fungos toxigênicos e por isso, merecem especial atenção como uma forma alternativa de descontaminação. Já a vantagem do gás ozônio, além de ser reconhecido como seguro e não deixar resíduos, demostra ter potencial para degradação das micotoxinas e dos resíduos de agrotóxicos.

Considerando a constante presença de fungos, micotoxinas e resíduos de agrotóxicos nos grãos, este estudo busca estratégias para o controle e descontaminação, investigando a eficiência e o mecanismo de ação dos tratamentos propostos frente a estes contaminantes. Vale ressaltar que o trigo produzido no Brasil é consumido pela população brasileira, neste sentido, perdas quantitativas na nossa produção devido a qualidade inferior dos grãos pela presença destes contaminantes, terão que ser supridas com maiores importações de outros países. Sendo que

muito destes não possui legislação específica para micotoxinas em trigo. Todas estas ações tem o objetivo maior de incentivar o aumento da qualidade e segurança dos produtos para consumo, já que a presença destes contaminantes nos grãos de trigo é reportada em todo o mundo.

2 OBJETIVOS

2.1 GERAL

Estudar estratégias de controle e descontaminação do trigo (*Triticum aestivum* L.) em grãos com relação a fungos, micotoxinas e agrotóxicos, utilizando compostos químicos e ozônio gasoso.

2.2 ESPECÍFICOS

- Verificar a umidade e atividade de água, a presença de espécies fúngicas e a micotoxina deoxinivalenol (DON) do trigo em grãos no período de pós-colheita.
- Avaliar a eficiência de descontaminação e o mecanismo de ação das nanopartículas de ouro frente a importantes fungos toxigênicos do trigo em grãos.
- Avaliar a eficiência de descontaminação e o mecanismo de ação de compostos inorgânicos: zinco e nanopartículas de zinco, frente a fungos (*Fusarium graminearum*, *F.verticillioides*, *Aspergillus flavus*, *Penicillium citrinum*) e a produção de micotoxinas (DON, fumonisinas - FBs, aflatoxinas - AFLs, citrinina - CTR) frequentemente encontradas em grãos de trigo.
- Aplicar compostos de zinco nas plantas de trigo na época de floração e avaliar a eficiência como agente anti-fúngico para *F. graminearum* e anti-micotoxigênico para DON.
- Avaliar a eficiência de descontaminação e o mecanismo de ação do gás ozônio frente a fungos toxigênicos (*F. graminearum*, *F. verticillioides*, *P. citrinum*, A. *flavus* e *A. parasiticus*) frequentemente encontrados no trigo em grãos.
- Aplicar ozônio gasoso no trigo em grãos em silos de armazenagem e avaliar a descontaminação de fungos (*F. graminearum*, *A. parasiticus*, *P. citrinum*) e micotoxinas (DON, AFLs, CTR).
- Avaliar possíveis alterações físicas e bioquímicas no trigo em grãos após descontaminação por ozônio gasoso nas concentrações que foram eficientes para a inibição de fungos e micotoxinas.
- Aplicar ozônio gasoso no trigo em grãos em silos de armazenagem e avaliar a descontaminação dos resíduos de agrotóxicos deltametrina e fenitrotiona (inseticidas).

CAPÍTULO 1
REVISÃO BIBLIOGRÁFICA

3 REVISÃO BIBLIOGRÁFICA

3.1 TRIGO

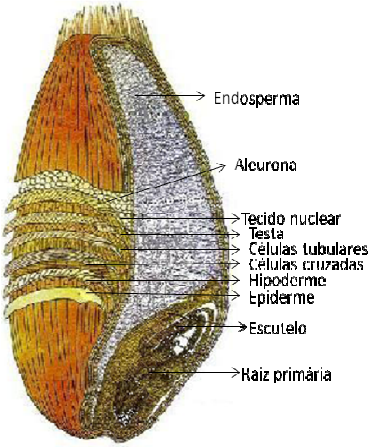
3.1.1 Características da planta

O trigo é uma gramínea do gênero *Triticum*, cultivada durante o inverno e a primavera. Há muitas variedades de trigo que diferenciam-se pela produtividade, conteúdo de farinha no grão, teor de nutrientes, resistência a doenças ou adaptação do clima e solo. Três espécies são destacadas pela representatividade de mais de 90% do trigo cultivado no mundo: *Triticum aestivum*, *Triticum compactum* e *Triticum durum*, sendo que o *T. aestivum* é o mais cultivado, respondendo por mais de quatro quintos da produção mundial. *T. aestivum* possui cerca de 15% de proteína, sendo a principal o glúten, importante para o processo de panificação. *T. compactum* tem menor teor de glúten e é utilizado para a fabricação de biscoitos e bolos mais macios e menos crocantes. Já o *T. durum* é indicado para massas e macarrão, pois essa espécie forma um glúten mais resistente, permitindo uma textura firme após o cozimento (ABITRIGO, 2013; EMBRAPA, 2013).

Os grãos de trigo podem ser divididos em três partes distintas: endosperma (83%), pericarpo (farelo - 14%) e germe (3%). O endosperma inclui o amiláceo e a camada de aleurona. O pericarpo consiste de pelo menos seis tecidos diferentes e o gérmen inclui o escutelo e o embrião. O pericarpo é a parte mais externa do grão e é rico em pentosanas, celulose, cinzas e proteína. Já a semente é formada pelo endosperma (maior depósito de energia do grão) e o gérmen, que é o tecido vivo responsável pela germinação, contendo alto conteúdo de proteína, lipídios, açúcares redutores e cinzas (Figura 1). Do ponto de vista botânico, a aleurona é parte do endosperma, mas no processo de moagem faz parte do farelo, é uma camada rica em cinza (fósforo), proteína, lipídios, vitaminas (niacina, tiamina, riboflavina) e enzimas (GERMANI et al., 1993; IAPAR, 2001).

Com relação aos valores nutricionais dos grãos de trigo, na Tabela 1 são apresentadas com maior descrição os dados nutricionais do farelo e farinha do trigo integral, os quais constituem altas fontes de carboidratos.

Figura 1 - Partes do grão de trigo



Fonte: Abitrigo, 2005

Tabela 1 - Valores nutricionais do farelo e farinha de trigo integral com os principais componentes

TRIGO	FARELO	FARINHA	TRIGO	FARELO	FARINHA
Componentes	**Valores por 100g**		**Vitaminas**	**Valores por 100g**	
Água	9,89 g	10,74 g	Tiamina	0,523 mg	0,502 mg
Energia	216 kcal	340 kcal	Riboflavina	0,577 mg	0,165 mg
Proteínas	15,55 g	13,21 g	Niacina	13,578 mg	4,957 mg
Lipídeos	4,25 g	2,50 g	Vitamina B6	1,303 mg	0,407 mg
Carboidratos	64,51 g	71,97 g	Folato	79 µg	44 µg
Fibras	42,8 g	10,7 g	Vitamina A e K	9 UI e 1,9 µg	9 UI e 1,9 µg
Açúcar	0,41 g	0,41 g	Vitamina E	1,49 mg	0,71 mg
Minerais			**Lipídeos**		
Cálcio	73 mg	34 mg	Total saturado	0,630 g	0,430 g
Ferro	10,57 mg	3,60 mg	Total monoinsaturado	0,637 g	0,283 g
Magnésio	611 mg	137 mg	Total poliinsaturado	2,212 g	1,167 g
Fósforo	1013 mg	357 mg	-	-	-
Potássio	1182 mg	363 mg	-	-	-
Sódio	2 mg	2 mg	-	-	-
Zinco	7,27 mg	2,60 mg	-	-	-

Fonte: USDA, 2014

3.1.2 Produção do trigo

A produção de trigo é uma das mais importantes atividades agrícolas para a alimentação humana e animal em todo o mundo, representa a segunda maior cultura de grãos em produção, sendo

superado apenas pelo milho. Cerca de 21% dos alimentos dependem da cultura do trigo que cresce em 200 milhões de hectares de terra agrícola em todo o mundo. Mesmo que os países em desenvolvimento sejam grandes importadores de trigo (43% das importações de alimentos), 81% do trigo é consumido pelo mesmo país que o produz. Nos anos anteriores a 2020, a expectativa é que nos países em desenvolvimento, a demanda de trigo para consumo humano aumente em 2,6% ao ano. Sendo assim, o rendimento médio global do trigo terá que aumentar nos próximos anos de 2,6 a 3,5 toneladas ha^{-1} (CIMMYT, 2005).

Nos últimos 30 anos, a área de trigo no Brasil tem oscilado significativamente, devido a políticas econômicas inconsistentes e influência de condições climáticas adversas, principalmente na região Sul. A estimativa de safra da Conab realizada em dezembro de 2012 apontou para uma produção de trigo de 4.300,4 mil ton contra 5.788,6 mil de ton, nas safras de 2011/12, representando uma redução de 25,7%, da ordem de 1.488,2 mil ton. Esta redução ocorreu por causa do recuo na produtividade da cultura no Rio Grande do Sul e em Santa Catarina, devido a problemas climáticos. Já na safra 2012/13, a produção de trigo foi de 4.379,5 mil ton. Nesta safra, a região Sul foi responsável pela produção de 4.148,9 mil ton, representando cerca de 94,7% da produção de trigo no Brasil. Sendo o Estado do Paraná responsável por 2.112,50 mil ton, superior a do Rio Grande do Sul com 1.894,80 mil ton e Santa Catarina com 141,60 mil ton. Considerando a safra de 2013/14, a produção de trigo no Brasil voltou a aumentar, com 5.555,80 mil ton, sendo a região Sul com a produção de 5.252,50 mil ton (95%). Nesta safra, os estados de Paraná, Santa Catarina e Rio Grande do Sul foram responsáveis por 2.624,50, 173,20 e 2.455,30 mil ton, respectivamente (CONAB, 2013).

O consumo anual de trigo no país tem se mantido em torno de 10 milhões de ton, sendo que a demanda atual de trigo importado de aproximadamente 5,0 milhões ton. Os principais fornecedores responsáveis por suprir a demanda de trigo no Brasil são a Argentina (84%), EUA (7%), Polônia (6%), Canadá (2%) e outros países (1%) (CONAB, 2012). As grandes regiões produtoras de trigo no mundo se concentram nas zonas de clima temperado. O excesso de chuvas, aliado à temperaturas elevadas, favorecem o aparecimento de pragas e contaminantes no trigo. Os grãos e sementes podem estar expostos a deterioração tanto no campo quanto na armazenagem, principalmente pelo crescimento de fungos e pelos efeitos advindos das estocagens de grandes quantidades de alimentos em depósitos inadequados (úmidos e quentes). No Brasil, a estimativa de perda quantitativa corresponde à

média anual de 20% dos grãos armazenados, podendo chegar à perda total em alguns armazéns (ZYLBERSZTAJN et al., 2004), prejudicando ainda mais a demanda do consumo interno.

3.1.3 Etapas de produção do trigo

3.1.3.1 Pré-produção

A escolha das cultivares devem levar em conta as características climáticas de cada região, bem como, as características da propriedade de cultivo e da cultivar. A indicação para o período de semeadura segue o estabelecido pelo Zoneamento Agrícola do Ministério da Agricultura, Pecuária e Abastecimento (MAPA) para a cultura de trigo. O controle de semeadura deve ser usada para buscar a colocação adequada de sementes e de fertilizantes no solo, segundo densidades e espaçamentos recomendados para a cultivar que se deseja estabelecer (PEREIRA, 2009).

O cultivo isolado e repetido de uma mesma espécie na mesma lavoura propicia o aumento dos prejuízos causados por determinadas doenças (que causam podridões radiculares, manchas foliares, grãos danificados, etc.), pragas e plantas daninhas associadas, as quais reduzem o rendimento, a qualidade e a lucratividade. Por isso, o planejamento de um sistema de rotação de culturas é fundamental na produção agrícola de culturas como o trigo (PEREIRA, 2009).

3.1.3.2 Produção

Em relação ao plantio do trigo, os principais problemas climáticos são dependentes de cada região: nas temperadas, o excesso de umidade relativa em setembro/outubro, geada no espigamento, chuva na colheita e granizo são os principais problemas; já na região subtropical destacam-se a umidade relativa elevada, geada e seca no espigamento, bem como chuva na colheita; e por fim, na região tropical, são relatados umidade relativa elevada no verão e temperatura do ar elevada durante o período de enchimento dos grãos. O cultivo do trigo pode ser prejudicado por estas condições ambientais no campo, sendo que a germinação das sementes maduras na espiga pode coincidir com condições desfavoráveis, como a precipitação antecipada e alta umidade, o que reduz o rendimento e a qualidade do alimento (EMBRAPA, 2013). Estas condições no campo permitem maior proliferação de fungos e deterioração dos grãos.

Perdas quantitativas de grãos também são comuns no momento da colheita por problemas técnicos de maquinaria, perdendo em média 5% dos grãos de trigo. Na prática, a regulagem da colhedora deve ser observada, sendo que à medida que a colheita é processada as condições de umidade do grão e da palha variam e a colhedora necessitará de novas regulagens de rotação e velocidade (Figura 2) (EMBRAPA, 2013).

Figura 2 - Colheita do trigo mecanizada

Fonte: KURTZ, 2008a

3.1.3.3 Pós-produção

O teor de umidade recomendado para armazenar o trigo colhido é de 13%. Para o transporte do trigo em caminhões, a umidade também não deve exceder ao limite permitido. Em condições em que o trigo colhido apresentar umidade superior à indicada para armazenamento, este deve ser submetido à secagem. Em lotes com mais de 16% de umidade, recomenda-se a secagem lenta para evitar danos físicos no grão. Entretanto, a secagem do trigo é uma operação crítica que pode alterar significativamente a qualidade dos grãos, se realizada sem cuidados. A secagem artificial de grãos em curto período de tempo caracteriza-se pela movimentação de grandes massas de ar aquecidas até atingirem temperaturas na faixa de 40 a 60 ºC na massa de grãos (PORTELLA, 2009).

No armazenamento, os silos devem ser limpos e secos. Além disso, deve ser monitorado durante todo o armazenamento, para acompanhar a ocorrência de pragas e insetos que podem atacar a massa de grãos, e controlar umidade e temperatura que podem influenciar na

conservação do trigo armazenado (Figura 3). A técnica de manejo de pragas é utilizado na unidade armazenadora de grãos para evitar perdas ocasionadas pelas pragas em armazéns. O tratamento com inseticidas químicos também faz parte da proteção dos grãos contra as pragas e pode ser realizado no momento de abastecer o armazém ou na forma de pulverização na correia transportadora, ou outros locais de movimentação dos grãos (TIBOLA et al., 2009a). Em condições ambientais desfavoráveis à atividade metabólica do grão, a alta umidade e temperatura podem propiciar o desenvolvimento de fungos produtores de micotoxinas nocivas ao homem e aos animais (LORINI, 2009).

Figura 3 - Silo graneleiro para armazenamento de grãos

Fonte: KURTZ, 2008b

3.2 PRINCIPAIS CONTAMINANTES DO TRIGO

Os principais contaminantes dos grãos de trigo são os insetos, fungos, micotoxinas e agrotóxicos. As condições climáticas durante o crescimento das plantas é um fator chave na formação de fungos e produção de micotoxinas. Períodos prolongados, 48 a 72 horas de alta umidade e temperatura quentes, 25 a 35ºC, são fatores favoráveis para o crescimento destes micro-organismos. Além destes fatores, outros como danos mecânicos, danos por insetos, condições inadequadas de secagem e armazenamento dos grãos de trigo também irão influenciar no crescimento dos fungos (LLORENS et al., 2004; MATEO; MATEO; JIMÉNEZ, 2002).

3.2.1 Insetos

Os insetos-praga ocasionam danos aos grãos de trigo e tem relação direta com outras contaminações como a proliferação de fungos e a produção de micotoxinas. Devido sua atividade metabólica há um aumento do teor de umidade e temperatura no interior da massa de grãos, o que agrava o desenvolvimento de fungos. A presença de fragmentos de insetos, nos produtos finais, também causa expressivos prejuízos para a cadeia produtiva (LORINI, 2008). Os insetos podem ser classificados como pragas primárias e secundárias. As pragas primárias são aquelas que atacam grãos inteiros e sadios e podem ser denominadas pragas primárias internas ou externas. As primárias internas perfuram e penetram nos grãos para completar seu desenvolvimento. Alimentam-se de todo o interior do grão e possibilitam o desenvolvimento de outros agentes de deterioração dos grãos. Pragas primárias com uma maior ocorrência no trigo são as espécies *Rhyzopertha dominica* e *Sitophilus oryzae*. As pragas primárias externas destroem a parte exterior do grão (casca) e alimentam-se da parte interna, no entanto, não se desenvolvem no interior do grão. Exemplo desta praga é a traça *Plodia interpunctella*. As pragas secundárias são aquelas que não conseguem atacar grãos inteiros, pois requerem que os grãos estejam danificados ou quebrados para se alimentarem. Essas pragas ocorrem na massa de grãos quando estes estão trincados, quebrados ou mesmo danificados por pragas primárias. Multiplicam-se rapidamente e causam prejuízos elevados. As principais espécies são: *Cryptolestes ferrugineus*, *Oryzaephilus surinamensis* e *Tribolium castaneum* (LORINI, 2008).

3.2.2 Fungos

Os fungos são os grandes responsáveis pela deterioração em partes da planta, grãos e sementes durante e após a colheita. Na armazenagem são responsáveis pelo aquecimento dos grãos e levam a perda do poder germinativo, descoloração, redução do valor nutritivo e alterações no odor podendo afetar a qualidade dos produtos derivados comercializados (SCUSSEL; BEBER; TONON, 2011).

Alguns fungos toxigênicos, principalmente do gênero *Aspergillus*, *Penicillium* e *Fusarium* podem se desenvolver tanto nas plantas no campo, quanto no período de armazenamento dos grãos. *Fusarium* sp. podem ser mais facilmente encontrado como contaminantes dos grãos no campo, sendo que *Aspergillus* sp. e *Penicillium* sp. podem se desenvolver durante armazenagem dos grãos em condições inadequadas.

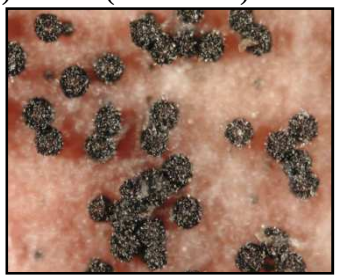
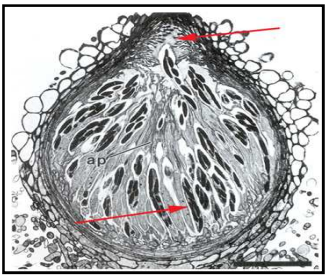
As espécies fúngicas encontradas no trigo podem trazer prejuízos a planta, aos grãos e sementes, pois em condições naturais são capazes de produzir metabólitos secundários tóxicos, as micotoxinas. Estes compostos são biossintetizados e excretados para o alimento onde o fungo cresce (CAST, 2003). A presença destes fungos toxigênicos é indicativo de que há ocorrência de micotoxinas no alimento, principalmente aqueles contendo cereais, leguminosas e algumas frutas, como maçã, uva e figo (SCUSSEL; BEBER; TONON, 2011). As micotoxinas podem causar vários danos ao organismo humano e animal, principalmente, nas funções hepáticas, renais, digestivas, neurológicas e circulatórias, muitas vezes levando ao desenvolvimento de tumores, podendo inclusive ser letal (BENNETT; KLICH, 2003).

3.2.2.1 Gênero *Fusarium*

O gênero *Fusarium* é encontrado no trigo principalmente em período de pré-colheita, no campo. As espécies mais comuns encontradas são *F. graminearum*, *F. verticillioides*, *F. culmorum* e *F. avenaceum* (CALORI-DOMINGUES et al., 2007). Em trigo colhido na Argentina, o gênero *Fusarium graminearum* foi o mais frequente (GONZALES et al., 1996; 1999). Na Quênia, as principais espécies que foram identificadas no trigo foram: *F. oxysporum* (15.5%), *F. graminearum* (10.6%), *F. chlamydosporum* (8.3%), *F. verticillioides* (7.6%), *F. avenaceum* (6.6%) e *F. sporotrichioides* (3.5%) (MUTHOMI et al., 2008).

O trigo no campo pode ser afetado por uma doença chamada giberela do trigo, causada pela espécie *F. graminearum* (teleomorfo *Gibberella zeae*). O gênero *Gibberella* caracteriza-se por apresentar peritécios de coloração brilhante e que frequentemente se formam em um estroma (estruturas somáticas onde os corpos de frutificação se desenvolvem). Os peritécios de *G. zeae* são de coloração negra-azulada na maturação e permanecem sobre os restos culturais entre uma estação de cultivo e outra (LIMA, 2004; PANISSON; REIS; BOLLER, 2002) (Figura 4a). No interior dos peritécios estão localizados os ascos que irá conter os ascósporos (esporos sexuais). Estes esporos serão forçadamente liberados do peritécio através do ostíolo (Figura 4b). Desta forma, esses esporos são transportados pelo vento a longas distâncias e podem ser depositados sobre anteras da planta, causando infecção (SCHMALE; BERGSTROM, 2003).

Figura 4 - (a) Peritécios negro-azulados de *Giberella zeae*; (b) Seção transversal de um peritécio de *Giberella zeae* apresentando ostíolo (seta acima) e ascos (seta abaixo)

(a)  (b) 

Fonte: SCHMALE; BERGSTROM, 2003 e TRAIL; COMMON, 2000

A giberela no trigo é uma doença que ataca as espigas, causando despigmentação das espiguetas afetadas. Dependendo das condições climáticas e da cultivar, pode ser observado um micélio branco a rosado sobre as espiguetas (Figura 5a). Esta doença pode comprometer toda a produção quando a floração ocorre em épocas com temperaturas superiores a 20ºC e a duração do molhamento das espigas é superior a 72h (SCHMALE; BERGSTROM, 2003). Os grãos produzidos são chochos, enrugados e de coloração branco a rosada (Figura 5b). Esta doença é reportada mundialmente e está associada a contaminação de deoxinivalenol (DON) nos grãos de trigo. DON tem gerado preocupações crescentes devido ao seu potencial em causar efeitos tóxicos sobre a saúde animal e humana. A infecção por giberela na planta resulta em perdas econômicas diretas, derivadas da redução na produção e perda de grãos, além de perdas indiretas, resultantes da contaminação por micotoxinas, que levam a rejeição ou desvalorização dos grãos no mercado. Sua ocorrência tem aumentado e atingido níveis epidêmicos em vários países. Na China e no Japão, existem relatos de até 50% de perdas associadas à giberela em trigo, enquanto na Argentina foram estimadas perdas de 30% na produção (LIMA, 2002). Em vários países, como México, Paraguai, Austrália, também há relatos de perdas causadas por giberela em trigo. A doença vem assumindo destacada expressão econômica nos Estados Unidos da América e no Canadá em trigo e em cevada, com perdas substanciais (LIMA, 2002). No Brasil, em 1998, nos ensaios de rendimento de trigo conduzidos na Empresa Brasileira de Pesquisa Agropecuária (Embrapa Trigo), constatou-se, em genótipos brasileiros mais suscetíveis, aproximadamente 40% de grãos com sintomas de giberela. No Brasil, esta doença tem sido estudada há

mais de três décadas e estudos recentes indicam que a doença, que se apresentava na forma de epidemias leves e esporádicas, alcançou o status de principal doença nas regiões tritícolas com maior frequência, principalmente no Sul do Brasil, causando muitos impactos econômicos (EMBRAPA, 2013; LIMA, 2002).

Figura 5 - (a) Espiga de trigo com giberela causada por *Gibberella zeae*, com crescimento micelial de coloração branco a rosada e (b) grãos de trigo giberelados

(a)  (b) 

Fonte: SANTANA; CHAVES, 2009 e autor (2014)

3.2.2.2 Gênero *Aspergillus*

No trigo, muitas espécies toxigênicas do gênero *Aspergillus* podem ser encontradas, especialmente no armazenamento, entre as principais estão: *A. flavus*, *A. niger*, *A. ochraceus*, *A. versicolor* e *A. terreus*. Riba e colaboradores (2008) encontraram a predominância do gênero *Aspergillus* (81.8%) em trigo armazenado por 6 meses em silos na região da Argélia. As principais espécies foram *A. flavus* (35%), *A. niger* (22.3%), *A. ochraceus* (4.5%), *A. versicolor* (10%) e *A. terreus* (19%). O gênero também apresentou predominância (64.5%) em amostras de grãos de trigo armazenados em silos coletados em outro estudo, sendo as espécies mais encontradas: *Aspergillus* section Nigri (26%), Circumdati (26.2%) e Terrei (28.7%) (RIBA et al., 2010).

3.2.2.3 Gênero *Penicillium*

O gênero *Penicillium* também pode ser comumente encontrado em trigo em grãos armazenados em condições inadequadas. As principais espécies encontradas são *P. verrucosum*, *P. citrinum* e *P. oxalicum*. Em grãos de trigo coletado de Buenos Aires, Argentina, o

gênero *Penicillium* (42%) foi o mais encontrado, seguido de *Fusarium* (27%) e *Aspergillus* (12%) (ROIGÉ et al., 2009). Em estudo na Austrália, o gênero *Penicillium* também foi o mais encontrado em amostras de trigo (BERGHOFER et al., 2003).

A Figura abaixo mostra grãos sadios (Figura 6a) e grãos contaminados (Figura 6b) com fungos toxigênicos dos gêneros *Aspergillus* e *Penicillium* devido a alta umidade (90%) e temperatura (30ºC).

Figura 6 - (a) Grãos de trigo sadios e (b) Grãos de trigo contaminados com fungos toxigênicos

(a)  (b) 

Fonte: autor (2014)

3.2.3 Micotoxinas

O desenvolvimento das micotoxinas depende do tipo de alimento, bem como das variações climáticas, condições de colheita e/ou armazenagem. Para trigo, as principais micotoxinas que podem ser encontradas são DON, fumonisinas (FBs), toxina T_2 (T_2), zearalenona (ZON), aflatoxinas (AFLs), citrinina (CTR) e ocratoxina A (OTA) (SCUSSEL; BEBER; TONON, 2011). A Tabela 2 apresenta vários estudos na literatura que relatam a ocorrência mundial das principais micotoxinas encontradas em grãos de trigo.

Tabela 2 - Ocorrência mundial das principais micotoxinas encontradas em grãos de trigo

(Continua)

País		Frequência (%)	Min e Max. (µg/kg)	Média (µg/kg)	Referências
Deoxinivalenol (DON)					
Argentina		37,4	71,5 - 505	201,3	QUIROGA et al., 1995
Argentina		78	380 - 5330	2100	LORI et al., 2003
Quênia	Nakuru	75	105 - 303	132,70	MUTHOMI et al., 2008
	Niandarua	59	105 - 289	113	
Tunísia		83	12800 - 30500	21520	BENSASSI et al., 2010
Sérvia	2005*	85,7	52 - 3306	605,52	STANKOVIC et al., 2012
	2007*	93,3	50 - 1090	282,84	
Malásia		25	5,5 - 18,62	-	SOLEIMANY et al. 2012
Brasil		66,4	206,3 - 4732,3	1894,90	SANTOS et al., 2013
Índia		40	70 - 4730	910	MISHRA et al., 2013
Marrocos		11,1	321 - 1310	502,10	ENNOUARI et al., 2013
Argentina		96	50,60 - 28650	3211	CENDOYA et al., 2014
Toxina T2					
Argentina		7,7	12,7	165,3	QUIROGA et al., 1995
Quênia	Nakuru	68	20 - 60	22,7	MUTHOMI et al., 2008
	Niandarua	86	20 - 66	29,5	
Sérvia	2005	75	60 - 495	171,52	STANKOVIC et al., 2012
	2007	60	86 - 200	86,75	
Malásia		15	5,95 - 34,45	-	SOLEIMANY et al., 2012
Fumonisinas (FBs)					
Espanha		47,1	200 - 8,800	2900	CASTELLÁ; BRAGULAT; CABAÑES et al., 1999
Malásia		15	12,15 - 29,35	-	SOLEIMANY et al., 2012
Sérvia	2005	82,1 (FB_1)	750 - 5400	2079,45	STANKOVIC et al., 2012
	2007	92 (FB_1)	750 – 4900	918,76	
Argentina	(FB_1)	97	0,16 - 680,44	30,07	CENDOYA et al., 2014
	(FB_2)	51	0,25 - 23,67	1,47	

Tabela 2 - Ocorrência mundial das principais micotoxinas encontradas em grãos de trigo

(conclusão)

País		Frequência (%)	Min e Max. (µg/kg)	Média (µg/kg)	Referências
Zearalenona (ZON)					
Quênia	Nakuru Niandarua	60 53	1,6 - 35 1 - 96	3,8 7,1	MUTHOMI et al., 2008
Sérvia	2005 2007	88,6 94,6	10 - 143 16 - 201	19,74 29,01	STANKOVIC et al., 2012
Tunísia		79,3	0 - 560	110	ZAIED et al., 2012
Malásia		20	1,42 - 12,74	-	SOLEIMANY et al., 2012
Aflatoxinas (AFLs)					
Turquia		59	0,01 - 0,64	-	GIRAY et al., 2007
Quênia	Nakuru Niandarua	41 52	0 - 7 2 - 7	1,7 2,2	MUTHOMI et al., 2008
Argélia		83,3	0,31 - 4,62	-	RIBA et al., 2010
Malásia		75	0,2 - 3,2	-	SOLEIMANY et al., 2012
Paquistão		20	1,8 - 15,5	6,6	LUTFULLAH; HASSAIN, 2012
Citrinina (CTR)					
Argentina		100	65 - 460	-	COMERIO; PINTO; VAAMOND, 1998
Tunísia		50	1 - 170	28	ZAIED et al., 2012
Russia		5,9	20 - 1000	-	KONONENKO; BURKIN, 2013
Ocratoxina (OTA)					
Reino Unido		98,5	20 - 600	-	PRICKETT; MACDONALD; WILDEY, 2000
Argélia		45	0,21 - 3,91	-	RIBA et al., 2008
Malásia		30	0,15 - 2,11	-	SOLEIMANY et al., 2012

Fonte: autor (2014)
Nota: * ano do estudo.

3.2.3.1 Micotoxinas produzidas por fungos mais comumente encontrados no trigo durante período de pré-colheita

3.2.3.1.1 Deoxinivalenol

É uma micotoxina do tipo B dos tricotecenos produzidas por *F. graminearum*, *F. culmorum* e *F. avenaceum*, entre outras espécies. Ambas espécies precisam de temperaturas ótimas para crescimento

(25ºC e 21ºC) e isso provavelmente afeta a distribuição geográfica. Esta toxina é resistente ao processo de moagem e aquecimento e, por isso, entra na cadeia alimentar de animais e humanos diretamente. É uma das micotoxinas consideradas mais importantes dos cereais, e são encontradas em todo o mundo como contaminantes de trigo, cevada, aveia, arroz, centeio e milho (MOSS, 2002).

Os principais efeitos toxigênicos de DON quando em doses baixas são diminuição de crescimento e anorexia, enquanto doses elevadas induzem vômitos, efeitos imunotóxicos e mudanças neuroquímicas no cérebro (WIJNANDS; VAN LEUSDEN, 2000). Efeitos tóxicos em animais tem sido bem documentados e focalizam principalmente o sistema imunológico e o trato gastrointestinal. Doses agudas são caracterizadas por efeitos como diarréia, vômito, leucocitose, hemorragia, choque circulatório, podendo levar a morte. As doses crônicas são caracterizadas por recusa alimentar, redução do ganho de peso e na absorção de nutrientes, além de alterações neuroendócrinas e imunológicas (LARSEN et al., 2004; PESTKA; SMOLINSKI, 2005). Estudos mais atuais tem demonstrado que o DON induz ao estresse oxidativo em ratos dependendo da dose utilizada (MISHRA, 2013; MISHRA et al., 2014) e que a produção de espécies reativas de oxigênio (EROs) pode estar associada a desregulação do sistema imunológico induzido por DON. Isso porque, o sistema imunológico é sensível ao DON e pode ser tanto estimulado, quanto suprimido, dependendo da dose e da frequência de exposição (PESTKA, 2010).

Entre os animais, os suínos são mais sensíveis ao DON do que aves, ratos e ruminantes, em parte por causa de suas diferenças no metabolismo, sendo os machos mais sensíveis que as fêmeas (DIEKMAN; GREEN, 1992; D'MELLO; PLACINTA; MACDONALD, 1999; ROTTER; PRELUSKY; PESTKA, 1996).

3.2.3.1.2 Toxina T2

Pertence ao tipo A dos tricotecenos produzidos por *F. sporotrichioides, F. poae, F. equiseti, F. Acuminatum* e *F. langsethiae* (CREPPY, 2002; RABIE et al., 1986). Estes fungos estão associados aos cereais tanto no campo, quanto após a colheita, no armazenamento dos grãos (ERIKSEN, 2003; LARSEN et al., 2004). O *F. sporotrichioides*, por exemplo, cresce a temperatura de 2-35ºC e com alta atividade de água (CREPPY, 2002). A temperatura ótima para a ocorrência de T-2 é relativamente baixa (8-14ºC) (RABIE et al., 1986),

esta toxina é geralmente encontrada em várias culturas de cereais, tais como trigo, milho, cevada, arroz, aveia e centeio (SCF, 2000).

Alimentos contaminados com T-2 podem causar efeitos graves na saúde humana e dos animais, podendo levar até a morte (HOLT et al., 1988; MOSS, 2002). Sinais gerais de toxicidade incluem náuseas, vômitos, tonturas, calafrios, dor abdominal, diarréia, perda de peso, necrose dérmica, aborto, inibição da síntese de proteínas, sendo tóxicos para os sistemas hematológico e linfático, produzindo imunossupressão. Danos no fígado e pulmões, além de alterações proliferativas do epitélio do esôfago e estômago em roedores também são relatados (MOSS, 2002; OMURTAG; YAZICIOGLU, 2001; PACIN et al., 1994).

3.2.3.1.3 Fumonisinas

As FBs são produzidas por diversas espécies do gênero *Fusarium*, especialmente por *F. verticillioides*, *F. proliferatum*, *F. nygamai*, além de *Alternaria alternata*. Estes fungos tem distribuição mundial, sendo considerados importantes patógenos de cereais em todas as fases de desenvolvimento, incluindo período de pós-colheita, durante armazenamento (DIAZ; BOERMANS, 1994). A FB_1 é a mais abundante e tóxica, representando 70% de contaminação total de alimentos naturalmente contaminados (MALLMAN et al., 2001).

As FBs são responsáveis pela leucoencefalomácia em equinos e coelhos, edema pulmonar e hidrotórax em suínos, efeitos hepatotóxicos, carcinogênicos e apoptose no fígado de ratos (POZZI et al., 2001). FBs são inibidoras específicas da ceramida sintase e seus efeitos tóxicos podem estar relacionados com sua capacidade em romper metabolismo de fosfolipídios, resultando em uma grande quantidade de problemas na regulação e comunicação celular. A *International Association on Research of Cancer* (IARC) classificou FBs e seus metabólitos como compostos de possível carcinogenicidade em humanos (IARC, 1993).

3.2.3.1.4 Zearalenona

A ZON é a micotoxina estrogênica mais estudada, é membro de um grande e variado grupo de compostos (estrogênicos) de estrutura semelhante e com propriedades farmacológicas. É não esteroidal e altamente termoestável, produzida por cepas de várias espécies do gênero *Fusarium*, incluindo *F. culmorum*, *F. equiseti*, *F. graminearum*, e *F. moniliforme*. Tal como acontece com outras micotoxinas, apenas algumas cepas de certas espécies podem produzir

ZON (ALLDRICK; HAJŠELOVA, 2004). Está associada principalmente com culturas de cereais e seus subprodutos. Ainda que esta é considerada uma micotoxina de campo, há evidências de que esta micotoxina também possa ser produzida em grãos já colhidos (ALLDRICK; HAJŠELOVA, 2004; ZINEDINE et al., 2007a).

ZON tem uma baixa toxicidade aguda oral, sendo que em termos de toxicidade subaguda ou subcrônica, os efeitos da ZON parecem refletir sua capacidade de se ligar aos receptores de estrogênio, levando a alterações no trato reprodutivo e uma variedade de sintomas, incluindo: diminuição da fertilidade, aumento da reabsorção embrioletal e redução da ninhada em animais, para humanos são poucos os dados disponíveis. A IARC concluiu que havia evidência limitada para avaliar a capacidade carcinogênica de ZON (IARC, 1993). Contudo, a *Scientific Committee on Food* (SCF) da União Européia observou a ocorrência de tumores em estudos clínicos devido às características estrogênicas da micotoxina (ALLDRICK; HAJŠELOVA, 2004; SCF, 2000; ZINEDINE et al., 2007a).

3.2.3.2 Micotoxinas produzidas por fungos mais comumente encontrados no trigo durante período de armazenamento

3.2.3.2.1 Aflatoxinas

As AFLs são caracterizadas pela ligação dihidrofurano ou tretrahidrofurano fundido a um anel de cumarina. Há mais de 20 derivados isolados de AFLs produzido por várias espécies de fungos, sendo que as mais importantes são produzidas por *A. flavus*, que produz AFB_1 e AFB_2 e *A. parasiticus*, que produz AFB_1, AFB_2, AFG_1 e AFG_2. Estas AFLs, assim como muitos outros compostos heterocíclicos, fluorescem e são distinguidos por suas propriedades de fluorescência. Ambos AFB_1 e AFB_2 formam fluorescência azul e AFG_1 e AFG_2 formam fluorescência verde-amarelada sob luz ultravioleta (HUSSEIN; BRASEL, 2001). Esses fungos sobrevivem em uma ampla gama de ambientes e podem ser encontrados no solo, em restos de plantas e animais, e em grãos e sementes. Estes dois fungos são responsáveis pela deterioração dos grãos armazenados em todo o mundo, sendo o *A. flavus* a principal causa de contaminação por AFLs no período pré-colheita de diversas culturas (HUSSEIN; BRASEL, 2001; LIU; GAO; YU, 2006).

A toxicidade das AFLs decresce na seguinte ordem: $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (SCUSSEL, 2002). Entre as aflatoxinas, AFB_1 é

a forma mais tóxica para os mamíferos e apresenta propriedades hepatotóxicas, teratogênicas e mutagênicas, é classificada no grupo classe 1 da IARC e causa danos tais como hepatite tóxica, hemorragia, edema, imunossupressão e carcinoma hepático (IARC, 1993; REDDY, 2008).

3.2.3.2.2 Citrinina

É produzida por espécies fúngicas dos gêneros *Aspergillus*, *Penicillium* e *Monascus*, foi isolada originalmente da espécie *Penicillium citrinum* (BLANC; LORET; GOMA, 1995). São encontradas como contaminantes em trigo, cevada, milho, centeio, aveia, arroz e amendoim. Fungos toxigênicos produtores de CTR podem estar presentes durante o processo de colheita, crescimento, transporte e de armazenamento dos cereais e seus produtos finais (MOLINIÉ et al., 2005).

CTR tem sido conhecida por ser nefrotóxico, hepatotóxico e cancerígeno para os seres humanos e animais. Quando suínos receberam CTR diariamente em doses orais de 20-40 mg/kg, ocorreram sinais de toxicidade como depressão, glicosúria e proteinúria. As lesões renais incluíam necrose e descamação das células do epitélio renal dos túbulos contorcidos proximais, dilatação dos túbulos, espessamento da membranas e proliferação de células no interstício (KROGH; HASSELAGER; FRIIS, 1970). Rovaris e Scussel (1998) verificaram que a CTR causou lesão renal em ratos caracterizado por presença de células epiteliais tubulares, no sedimento urinário, poliúria, proteinúria, glicosúria, hematúria, aumento da excreção de sódio, aumento da uréia e creatinina sérica, além da diminuição da creatinina endógena.

3.2.3.2.3 Ocratoxina A

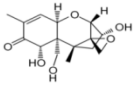
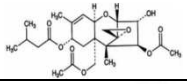
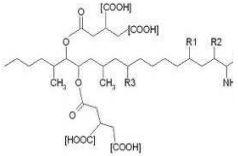
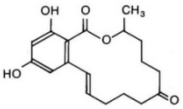
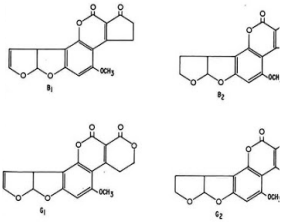
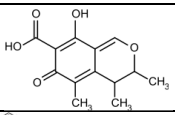
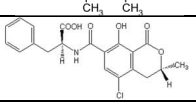
A estrutura química da OTA consiste de um 5'-cloro-3,4-dihidro fração-3-metil-isocumarina ligado à L-fenilalanina. Esta foi originalmente isolada de *Aspergillus ochraceus*. A OTA corresponde a um metabólito de espécies de *Aspergillus* e *Penicillium*, fungos capazes de crescer em climas e plantas diferentes, sendo que sua contaminação pode ocorrer em todo o mundo em diversas culturas de alimentos, inclusive cereais, produtos que representam a fonte dietética mais importante desta micotoxina, contribuindo em cerca de 50% da sua ingestão (AISH et al., 2004; DUARTE; PENA; LINO, 2010).

Os efeitos bioquímicos da OTA recorrem principalmente a partir de sua semelhança estrutural com o aminoácido essencial, fenilalanina. O principal efeito parece ser a inibição da síntese de proteínas, embora os efeitos secundários, tais como a inibição da síntese de RNA e DNA também têm sido implicados no seu mecanismo de ação (AISH et al., 2004). Esta micotoxina demonstra efeitos nefrotóxicos, teratogênicos, embriotóxicos, genotóxicos, neurotóxicos, imunossupressivos e carcinogênicos, sendo classificada como pertencente ao grupo 2B da IARC (DUARTE; PENA; LINO, 2010; IARC, 1993).

No Quadro 1 estão descritas as principais micotoxinas encontradas em trigo, estruturas químicas, fungos produtores, condições favoráveis para o crescimento e produção da micotoxina, e órgãos alvos da toxicidade.

As micotoxinas contaminantes do trigo podem ser resistentes ao processo de moagem e aquecimento, por isso, se presentes nos grãos de trigo podem permanecer em seus subprodutos. Estudos na literatura comprovam a presença das micotoxinas contaminantes do trigo em espaguete, macarrão, lasanha, talharim (IQBAL et al., 2014), farinha e farelo de trigo (BINDER et al., 2007; RIBA et al., 2010; RODRIGUES; NAEHRER, 2012) e pães de trigo (JUAN et al., 2008; ZINEDINE et al., 2007b). Devido a resistência ao processamento dos subprodutos de trigo, entram na cadeia alimentar de animais e humanos diretamente.

Quadro 1 - Principais micotoxinas encontradas em trigo, estruturas químicas, fungos produtores, condições favoráveis para o crescimento e produção da micotoxina, e órgãos alvos da toxicidade

Micotoxina	Estruturas químicas	Fungo	Condições favoráveis	Órgãos alvos toxicidade
Encontradas no trigo durante período de pré-colheita				
DON		*F. graminearum*	Temp.: 25ºC Aw: 0.88 – 0.99	Fígado (vomitoxina).
T-2		*F. graminearum* *F. poae*	Temp.: 25ºC Aw: 0.88 – 0.99	Sistemas hematológico e linfático.
FBs	 Fumonisina B_1: R1 = OH; R2 = OH; R3 = OH; Fumonisina B_2: R1 = OH; R2 = OH; R3 = H; Fumonisina B_3: R1 = H; R2 = OH; R3 = OH; Fumonisina B_4: R1 = H; R2 = OH; R3 = H;	*F. verticillioide*	Temp.: 25ºC Aw: 0.90 - 0.99	Sistema nervoso, causam neoplasias.
ZON		*F. graminearum*	Temp.: 25ºC Aw: 0.88 – 0.99	Sistema reprodutor.
Encontradas no trigo durante período de armazenamento				
AFLs		*A. flavus* *A. parasiticus*	Temp.: 30ºC Aw: 0.80 – 0.90	Fígado e rins, causam neoplasias.
CTR		*P. citrinum* *P. verrucosum* *A. ochraceus*	Temp.: 20-30ºC Aw: 0.88 – 0.99	Sistema renal e hepático.
OTA		*P. verrucosum* *A. ochraceus*	Temp.: 25ºC Aw: 0.97	Fígado e rins, causam neoplasias.

Fonte: adaptado de SOUZA KOERICH; SCUSSEL, 2012 e BOERMANS; LEUNG, 2007

3.2.4 Agrotóxicos

Para prevenir ou reduzir perdas na agricultura e melhorar a qualidade dos grãos, o uso de agrotóxicos é reconhecido como importante para a produção de alimentos. A utilização de agrotóxicos na cultura proporciona vantagens, tais como o aumento da eficiência, rentabilidade da produção e qualidade de cereais, no entanto, por outro lado, leva à contaminação da produção agrícola, a água, ar e solo (FERNANDEZ-ALVAREZ et al., 2008). Por isso, para alimentos de alta qualidade, além do controle de fungos e micotoxinas, os resíduos de agrotóxicos nos alimentos também devem ser monitorados. O uso incorreto e a exposição destes compostos podem contaminar o meio ambiente e a saúde dos homens e animais. A ingestão de alimentos é uma fonte toxicológica de exposição aos agrotóxicos cerca de 10^3 a 10^5 vezes maior que a ingestão de água pelos seres humanos (MARGNI et al., 2002). Devido ao nível de exposição do homem a estes compostos, podem ocorrer efeitos adversos como: dores de cabeça, náusea, distúrbios endócrinos e até câncer (COHEN, 2007).

A produção industrial de agrotóxicos é de 2,5 x 10^9 kg ao ano (CAJKA et al., 2008; KOVALCZUK et al., 2008). De acordo com os dados da Agência Nacional de Vigilância Sanitária (ANVISA) apresentados em 2009, o Brasil é o maior mercado consumidor de agrotóxicos no mundo, com 86% do uso destes produtos na América Latina, sendo o principal destino de agrotóxicos proibidos no exterior (BRASIL, 2009). Para melhor controle da exposição humana aos resíduos de agrotóxicos presentes nos alimentos, as agências reguladoras de vários países têm criado programas de monitoramento (BRASIL, 2009; ŠKRBIĆ; PREDOJEVIĆ, 2008).

No Brasil, a ANVISA criou em 2001, o Programa Nacional de Monitoramento de Resíduos de Agrotóxicos em Alimentos (PARA) com o objetivo de monitorar e adotar medidas de controle em relação aos níveis de resíduos de agrotóxicos, já que dados da Anvisa revelam que 15% dos alimentos consumidos pelos brasileiros apresentam taxas de resíduos de agrotóxicos em um nível prejudicial à saúde (BRASIL, 2003; BRASIL, 2009). As análises do PARA avaliam a quantidade de resíduo legalmente aceita dentro dos limites máximos de resíduos (LMR) na cultura e o uso indevido de agrotóxicos não permitidos. Os cereais mais analisados pelo PARA são o arroz e o feijão, no entanto, até o momento não há dados do PARA para trigo em grãos. De acordo com as metas estabelecidas em 2012, o trigo está entre as culturas a serem monitoradas no período de 2012 a 2015 (BRASIL, 2013b).

A preocupação de resíduos de agrotóxicos na cultura do trigo vem crescendo nas últimas décadas em decorrência de estudos que demonstram seus altos níveis nos alimentos, relacionando-os a graves problemas na saúde humana.

3.2.4.1 Agrotóxicos utilizados no trigo

Para o trigo, são utilizados durante a produção, os fungicidas e herbicidas para controlar doenças no trigo e plantas daninhas. Entretanto, esses em geral, são metabolizados dentro do intervalo de segurança e podem não permanecer resíduos nos grãos por ocasião da colheita. Já durante o período pós-colheita, a utilização de inseticidas organofosforados e piretróides para o controle de pragas de grãos armazenados é um dos métodos que preocupam quanto ao resíduo de agrotóxicos no trigo. O tratamento preventivo consiste na aplicação do inseticida via líquida sobre os grãos na correia transportadora, no momento do abastecimento do silo. Esse tratamento confere proteção contra a infestação por pragas, durante o armazenamento por períodos maiores de três meses. Entretanto, esses produtos apresentam restrições ao uso, devido aos problemas de persistência nos grãos e nos subprodutos na forma de resíduos, além da ocorrência de resistência das pragas aos inseticidas (EMBRAPA, 2013).

Os principais inseticidas para controle de pragas no armazenamento utilizados no trigo são do grupo químico piretróide (bifentrina e deltametrina) e organofosforados (fenitrotiona e pirimifós-metílico) (EMBRAPA, 2013).

A bifentrina é um inseticida caracterizado pela forte ação de choque que ocasiona no inseto, impedindo a sua alimentação. Possui mecanismo de ação baseado na interferência da ligação GABA e do ácido glutâmico nos sítios receptores, bloqueando a atividade neural. É aplicado ao se carregar os silos para armazenamento do trigo e apresenta ação especialmente contra: *Sitophilus zeamais* (gorgulho) e *Rhizopertha dominica* (gorgulho dos cereais) que causam consideráveis danos a produção do alimento (AGROLINKFITO, 2012). A deltametrina também é um inseticida que possui semelhante mecanismo de ação. É aplicado nos grãos de trigo armazenados para combater: *Laemophloeus minutus* (besouro); *Tribolium castaneum* (besouro castanho); *Sitophilus oryzae* (gorgulho); *Rhizopertha dominica* (gorgulho dos cereais) e *Sitotroga cerealella* (traça) (AGROLINKFITO, 2012).

Inseticidas do grupo organofosforados utilizados no controle de pragas de grãos armazenado possui mecanismo de ação baseado em inibir permanentemente a enzima acetilcolinesterase através de sua fosforilação, causando acúmulo de acetilcolina e consequente superestimulação das terminações nervosas, tornando inadequada a transmissão de seus estímulos às células musculares, glandulares, ganglionares e do sistema nervoso central. Os principais utilizados são fenitrotiona para o controle de *Sitophilus oryzae* (gorgulho) e pirimifós-metílico para o controle de *Sitophilus zeamais* (gorgulho).

No Sul do Brasil, Kolberg (2008) estudou os níveis de resíduos em grãos de trigo e subprodutos em amostras coletadas em dois moinhos. Nos grãos de trigo encontrou níveis de 16.4 µg/kg de bifentrina e 158.8 µg/kg de pirimifós-metílico, entretanto, nenhuma esteve acima do LMR permitido pela legislação brasileira e internacional.

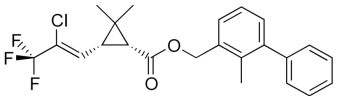
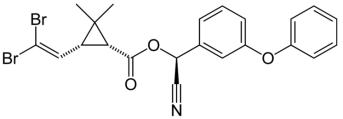
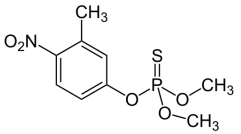
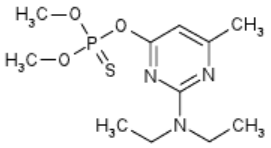
Sgarbiero (2001), analisou a ocorrência e degradação de resíduos de pirimifós-metílico em grãos de trigo e nos subprodutos: farinha branca e integral, pão e farelo. A farinha branca e integral no início do armazenamento apresentaram níveis semelhantes, em média de 2.744 e 2.497 mg/kg, respectivamente. O pão apresentou em média 2.627 mg/kg do agrotóxico, sendo o farelo, o subproduto que apresentou maiores níveis de resíduos, em média de 7.547 mg/kg. Após armazenamento, houve uma maior concentração de resíduos no farelo, em torno de 2,8 vezes quando comparada com os níveis detectados nos grãos. O pão preparado com a farinha branca apresentou aproximadamente 50% de redução nos níveis de resíduos. Ao final do período de armazenamento, 240 dias, a farinha integral apresentou concentração de resíduos 60% superior, quando comparada com a farinha branca.

Na África do Sul, o trigo importado foi encontrado com 30.8% das amostras contendo resíduos de fenitrotiona em níveis de 0.032 à 0.445 mg/kg (DALVIE; LONDON, 2009). Nos EUA, 20.5% das amostras de trigo analisadas apresentaram resíduos múltiplos de agrotóxicos. Os mais freqüentes foram: clorpirifós, clorpirifós-metílico, cipermetrina, deltametrina, malation, pirimifós-metilíco, benomil, carbendazim e tiofanato-metílico (CEC, 2005).

No Cazaquistão na Ásia Central, de 45 amostras de trigo analisadas, 13 estavam com resíduos de agrotóxicos, os quais podemos destacar a deltametrina encontrada com níveis de 0,02 mg/kg (LOZOWICKA et al., 2014).

Além do monitoramento dos programas nacionais para levantamento de resíduos de agrotóxicos nos alimentos, alternativas que reduzam estes contaminantes seria de grande valia, já que é crescente a preocupação com os efeitos adversos destes compostos no meio ambiente e na saúde humana. No Quadro 2 estão descritos os principais agrotóxicos utilizados no trigo armazenado, estruturas químicas e toxicidade.

Quadro 2 - Principais agrotóxicos utilizados no trigo armazenado, estrutura química e toxicidade

Agrotóxico	Estruturas químicas	Toxicidade
Piretróides		
Bifentrina		Neurotóxicos. ✓ Ocorre disparos repetitivos devido ao aumento pós-potencial positivo (aumentando a frequência de abertura dos canais de sódio). ✓ Aumento da corrente desses íons para dentro do neurônio (despolarizando a membrana). ✓ Desta forma, diminuindo a amplitude do potencial de ação, com bloqueio da atividade neural.
Deltametrina		
Organofosforados		
Fenitrotiona		Inibem a colinesterase. ✓ Inibem principalmente a acetilcolinesterase, uma proteína molecular presente nas sinapses colinérgicas. ✓ Esta enzima é responsável pela hidrólise da acetilcolina em colina e ácido acético. ✓ A acetilcolina é uma substância transmissora de várias sinapses do sistema nervoso, transmitindo os impulsos nervosos em uma velocidade surpreendente de 1 a 2 milissegundos. ✓ Este agrotóxico inibe irreversivelmente a acetilcolinesterase, causando acúmulo da acetilcolina nas sinapses.
Pirimifós-metílico		

Fonte: AGROLINKFITO, 2012 e EMBRAPA, 2013

3.3 LEGISLAÇÃO

3.3.1 Micologia

Em relação aos parâmetros microbiológicos relativos à contagem padrão em placa, não há uma resolução nacional que estabeleça um limite máximo para fungos em cereais como os grãos de trigo. Entretanto, a presença de fungos neste alimento, especialmente fungos toxigênicos podem ser indicativos de deterioração e contaminação por micotoxinas.

Dependendo do tipo de processamento realizado com o trigo, farinhas, massas e produtos de panificação, a contagem padrão de fungos e leveduras pode ser bastante diversificada, pois seu desenvolvimento irá depender do tipo de substrato e das condições ambientais.

3.3.2 Micotoxinas

Devido aos efeitos graves que as micotoxinas podem causar nos seres humanos e animais, em muitos países, existe legislação nacional que estabelece limites máximos toleráveis (LMT) específicos para vários tipos diferentes de micotoxinas em uma variedade de alimentos. As micotoxinas mais comumente reguladas são AFLs, OTA, patulina (PAT), DON, ZON e FBs. Os LMT estabelecidos por legislações para uma ou mais micotoxinas variam de acordo com o país e características do alimento que é consumido, importado e/ou exportado (SCUSSEL; BEBER; SOUZA, 2010).

No Brasil, os limites para micotoxinas eram estabelecidos somente para AFLs por meio da Resolução N^{o} 34/76 da Comissão Nacional de Normas e Padrões para Alimentos, a qual estabelecia o limite aceitável de 30 µg/kg de AFLs para alimentos em geral. Em 2002, por meio da Resolução N^{o} 274/2002 da ANVISA este limite passou para 20 µg/kg. Contudo, em fevereiro de 2011 entrou em vigor uma nova Resolução (RDC N° 07, de 18 de Fevereiro de 2011) desenvolvida pela ANVISA que apresentou limites de aplicações imediatas e de aplicações previstas para os próximos anos pares, a partir de 2012, 2014 e 2016 (BRASIL, 2011) (Tabela 3). No entanto, em dezembro de 2013, uma nova legislação da ANVISA entrou em vigor, prorrogando o prazo limite para as próximas aplicações até 2017 (BRASIL, 2013a). Isto para permitir que os produtores de grãos e da indústria possam se adaptar à legislação, sem causar uma escassez de alimentos.

É mais completa que a versão anterior e contempla diversas classes de alimentos e várias das micotoxinas já anteriormente contempladas por outras legislações de referência, tais como as dos Estados Unidos e de países europeus (EU, 2006; FDA, 1993).

Tabela 3 - LMT para micotoxinas em trigo e produtos derivados

MICOTOXINA	ALIMENTO	LMT (µg/kg)
Aplicação (desde 2011)		
Aflatoxinas	Cereais (Trigo)	5
Ocratoxina A	Cereais (Trigo)	10
Aplicação (desde 2012)		
Deoxinivalenol	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo	2000
	Farinha de trigo, massas, crackers, biscoitos de água e sal, produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	1750
Zearalenona	Farinha de trigo, massas, crackers, produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	200
	Trigo integral, farinha de trigo integral e farelo de trigo	400
Aplicação (a partir de 2014, prazo limite até 2017)		
Deoxinivalenol	Trigo em grãos para posterior processamento	3000
	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo	1500
	Farinha de trigo, massas, crackers, biscoitos de água e sal, produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	1250
Zearalenona	Trigo em grãos para posterior processamento	400
Aplicação (a partir de 2016, prazo limite até 2017)		
Deoxinivalenol	Trigo integral, trigo para quibe, farinha de trigo integral, farelo de trigo	1000
	Farinha de trigo, massas, crackers, biscoitos de água e sal, produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	750
Zearalenona	Farinha de trigo, massas, crackers, produtos de panificação, cereais e produtos de cereais, exceto trigo e incluindo cevada malteada	100
	Trigo integral, farinha de trigo integral, farelo de trigo	200

Fonte: BRASIL, 2011 e BRASIL 2013a

A Tabela 4 apresenta a legislação de países internacionais como Estados Unidos e países europeus que apresentam LMT para micotoxinas em trigo e seus produtos derivados. Países do Mercosul, como Argentina e Uruguai, que são grandes exportadores de trigo para o Brasil, não possuem legislação vigente específica para o cereal.

Tabela 4 - LMT de países internacionais para micotoxinas em trigo e produtos derivados

MICOTOXINAS	PAÍS	ALIMENTO	LMT (µg/kg)
Aflatoxinas	Índia	Trigo	30
Ocratoxina A	Rússia	Trigo	5
	China	Cereais (Trigo)	5
Toxina T-2	Rússia	Grãos de trigo, farinha de trigo, massas	100
Deoxinivalenol	Canadá	Trigo	2000
		Trigo (alimentos para bebês)	1000
	China	Trigo	1000
	Índia	Trigo	1000
	Japão	Trigo	1100
	Rússia	Trigo, cereais de trigo, farinha de trigo, massas, produtos de panificação, gérmen de trigo	700
	USA*	Produtos de trigo acabados	1000
	UE**	Trigo	1750
		Cereais destinados a bebês	200

Fonte: EU, 2006; FDA, 1993
Nota: ***** USA - Estados Unidos da América
****** UE - União Européia

Diante da nova legislação da ANVISA, o mercado produtor brasileiro terá que se adequar ao LMT dos alimentos para micotoxinas. Novos estudos que possam eliminar/diminuir os níveis destas micotoxinas no trigo vem de encontro à necessidade do mercado atual para melhorar a qualidade e produção dos alimentos. Programas de monitoramento dos níveis de micotoxinas neste alimento são essenciais para estabelecer prioridades em ações de órgãos reguladores.

3.3.3 Agrotóxicos

O controle de resíduos de agrotóxicos em alimentos está baseado nos limites máximos de resíduos (LMRs) e no intervalo de segurança. Para garantir a segurança dos alimentos quanto ao nível de resíduos de agrotóxicos, o LMRs são definidos pela *Food and Agriculture Organization* (FAO), pela Comissão do Codex Alimentarius e pela *World Health Organization* (WHO), representando a concentração máxima de resíduo que poderá ser ingerida diariamente através da alimentação, prevenindo danos à saúde dos consumidores. No Brasil, a regulamentação do LMR e do intervalo de segurança dos

agrotóxicos para cada cultura é realizada pela ANVISA (BRASIL, 2009). Na Tabela 5 podemos verificar os LMRs estabelecidos para os inseticidas indicados para o trigo no período pós-colheita.

Tabela 5 - LMR para os inseticidas (mg/kg) em grãos de trigo e subprodutos

Inseticida	Trigo			Farinha		Pão	Farelo	Intervalo de Segurança
				Bran	Integral	Branco		
	Anvisa	Codex	UE	Codex		Anvisa	Codex	Anvisa
Bifentrina	0.6	0.5	0.5	0.2	-	-	2.0	30 dias
Deltametrina	1.0	-	2.0	0.3	-	-	5.0	30 dias
Fenitrotiona	1.0	-	0.5	2.0	5.0	0.2	200	120 dias
Pirimifós-metílico	10.0	-	5.0	5.0	-	-	15.0	30 dias

Fonte: BRASIL, 2009; CODEX, 2005; EMBRAPA, 2013; EU, 2005.

3.4 MEDIDAS PREVENTIVAS PARA CONTROLE DE CONTAMINANTES DO TRIGO

3.4.1 Pré-colheita

O monitoramento da cultura de trigo tem início no campo e é realizado como medida preventiva para evitar redução na produtividade das lavouras. A rotação de culturas é uma medida que minimiza infestações de pragas, patógenos e plantas daninhas, incrementa a fertilidade do solo, mantém a cobertura permanente do solo, minimizando a erosão e viabilizando o sistema de planto direto na palha. Para evitar o crescimento de plantas daninhas, a semeadura deve ser realizada sem a presença deste contaminante, com adubação correta, uso de sementes de qualidade e cultivares adaptadas, além dos níveis ótimos de densidade, época e profundidade da semeadura. Além deste modelo de produção, outras medidas preventivas são necessárias para o controle de doenças e pragas no trigo. O uso de fungicidas e inseticidas e de cultivares resistentes são as estratégias de controle mais utilizadas. O tipo de tratamento e aplicação (sementes, foliar, espiga), irá depender do tipo de doença ou praga que está afetando a cultura e da densidade populacional para identificar a necessidade de controle (SANTANA; CHAVES, 2009; TIBOLA et al., 2009b).

3.4.2 Pós-colheita

No armazenamento, o trigo deve ser monitorado para evitar pragas que atacam os grãos. Grãos danificados podem favorecer o desenvolvimento de fungos toxigênicos. Na Pós-colheita, os principais fatores que contribuem para o deterioração e contaminação dos grãos são a alta umidade e temperatura. Além das pragas e fungos toxigênicos, os resíduos de agrotóxicos também são contaminantes inerentes aos grãos principalmente neste período, pois se não respeitado o período de carência, podem chegar aos seus subprodutos (BRASIL, 2009).

Medidas preventivas devem ser adotadas para evitar danos causados por pragas no trigo armazenado. A técnica de manejo integrada de pragas deve ser seguida, realizando algumas medidas como: limpeza e higienização da unidade armazenadora; armazenamento do trigo com teor de umidade máxima de 13%; tratamento químico dos grãos específico para cada espécie-praga; monitoramento da massa de grãos, entre outros. O tratamento com inseticidas químicos protetores de grãos deve ser realizado no momento de abastecer o armazém e pode ser feito na forma de pulverização na correia transportadora ou em outros pontos de movimentação de grãos, para que ocorra uma perfeita mistura do inseticida com a massa de grãos. Durante o armazenamento, o trigo deve ser monitorado em todo o período para que qualquer irregularidade seja detectada rapidamente e medidas corretivas sejam adotadas (PORTELLA, 2009; TIBOLA et al., 2009a).

Medidas de controle também precisam ser adotadas para evitar a contaminação por fungos e micotoxinas nos grãos armazenados: secagem eficiente dos grãos no recebimento da unidade armazenadora, controle de umidade e temperatura no local da armazenagem e monitoramento dos grãos durante todo o período. Além disso, condições adequadas no período de colheita como: evitar danos mecânicos aos grãos e realizar a colheita no ponto de maturação correta, também são fatores importantes para evitar o crescimento de fungos durante o armazenamento (TIBOLA et al., 2009b).

Já para evitar a contaminação com resíduos de agrotóxicos em alimentos, o LMR e o intervalo de segurança dos agrotóxicos no trigo devem ser respeitados. Programas nacionais de monitoramento dos resíduos e contaminantes no trigo também são importantes para a inspeção e fiscalização, visando a segurança alimentar (BRASIL, 2009).

Mesmo que todas as medidas preventivas sejam adotadas, muitas vezes, não é possível ter total controle para evitar os contaminantes durante a produção do trigo, dessa forma, medidas de

descontaminação são necessárias. A obtenção de resultados de análise de contaminantes através de métodos rápidos e eficientes, torna possível a segregação de lotes conforme a exigência dos diferentes segmentos de mercado, de acordo com a qualidade tecnológica do grão. Entretanto, devido a presença destes contaminantes no trigo, ocorrem grandes perdas na produtividade do grão. Sabendo que a quantidade produzida no Brasil já é bastante reduzida em relação ao consumo interno, com as perdas na agricultura, maiores são os custos econômicos para importação do alimento.

Quando as medidas preventivas não são suficientes, o trigo pode estar contaminado por fungos toxigênicos e micotoxinas, incluindo os seus subprodutos, o que traz altos prejuízos a saúde humana e animal. Por isso, a descontaminação antes da utilização do alimento para fins de consumo humano ou animal é de fundamental importância. Portanto, métodos de descontaminação são de grande valia para evitar perdas na produção e melhorar a qualidade do trigo em grãos.

3.5 MÉTODOS DE DESCONTAMINAÇÃO DO TRIGO EM GRÃOS

A descontaminação do trigo pode ocorrer através da remoção do material contaminado ou destruição e degradação dos contaminantes, como fungos, micotoxinas e agrotóxicos. Pode ocorrer por agentes físicos (calor, micro-ondas, raios gama e luz ultravioleta), químicos (agentes oxidantes, ácidos, bases e ozonização) e biológicos (micro-organismos).

Qualquer que seja a estratégia de descontaminação, os seguintes critérios básicos devem ser seguidos: a micotoxina deve ser inativada ou destruída por transformação de compostos não tóxicos; esporos fúngicos e micélios devem ser destruídos, assim novas toxinas não são formadas (SCUSSEL, 2013); o alimento deve manter seu valor nutritivo e permanecer palatável após a descontaminação; as propriedades físicas da matéria-prima não devem alterar significativamente; e, por último, deve ser economicamente viável (PARK, 1993).

3.5.1 Descontaminação física

A classificação dos grãos é um tratamento físico de descontaminação, a eliminação de grãos quebrados, contendo esporos e a limpeza da superfície dos grãos é uma forma eficiente de reduzir as micotoxinas (BALZER et al., 2004). A limpeza dos grãos com água de

torneira sob pressão também reduz significativamente o conteúdo de micotoxinas (WILSON et al., 2004), entretanto, este tratamento somente é possível em alimentos antes da moagem por via úmida, pois o custo da secagem dos grãos é alto. O descascamento também é uma forma de reduzir a contaminação, entretanto a eficiência do processo irá depender da localização da micotoxina no grão (FANDOHAN et al., 2005; HOUSE; NYACHOT; ABRAMSON, 2003).

O tratamento térmico também pode ser utilizado, entretanto, a maioria das micotoxinas são estáveis ao calor e não são eliminadas totalmente com este tratamento (MEISTER; SPRINGER, 2004; VOSS et al., 2001). Além destes, a irradiação gama tem sido utilizada para reduzir a contaminação de esporos fúngicos. Neste tratamento, altas concentrações podem reduzir a capacidade de germinação dos grãos e podem ser pouco eficientes para alimentos já contaminados com micotoxinas (AZIZ; MOUSSA; FAR, 2004; NOROOZIAN et al., 1999).

3.5.2 Descontaminação biológica

Micro-organismos, especialmente bactérias e leveduras, têm sido estudados quanto à sua potencialidade para degradar micotoxinas ou reduzir a sua biodisponibilidade (EL-NEZAMI et al., 1998; 2002; FUCHS et al., 2008, HASKARD et al., 2001; PELTONEN et al., 2001). Durante o processo fermentativo utilizado na produção de pães a partir de grãos de trigo contaminados com DON, foi observada a redução dos níveis atribuída à fermentação e ao processo térmico ao qual o produto foi submetido. Esta descontaminação ocorreu devido à possibilidade da levedura adsorver as toxinas presentes, reduzindo a contaminação. Experimentos de fermentação alcoólica por *Saccharomyces cerevisiae* com mosto contaminado com DON e ZON mostraram resultados onde após 7-9 dias de fermentação o DON foi estável ao processo. Do conteúdo inicial de zearalenona, 69% foi convertido a β-zearalenol e 8.1% a α-zearalenol. A maior parte de metabolização da ZON ocorreu no 1º e 2º dias de fermentação, mostrando a instabilidade da toxina. Leveduras isoladas do intestino grosso e identificadas como gênero do membro *Trichosporon* também mostraram um potencial de desativação de ZON em alimentos para animais (MOLNAR et al., 2004). Outras espécies de leveduras como *C. intermedia*, *L. thermotolerans* e *C. friedrichii* apresentaram uma notável capacidade de adsorver OTA em suco de uva (FIORI et al., 2014).

Resultados com alta inibição na produção de AFLs utilizando micro-organismos como*: Bacillus spp* (98%), *A. flavus e A. parasiticus* (90%) e *Trichoderma* spp (75%) foram demonstrados em estudo de MALLMANN et al. (2006). Por outro lado, *Acinetobacter* sp. já mostrou ser eficiente para degradação de ZON (YU et al., 2011). Esta mesma micotoxina também foi inativada por duas estirpes de *Rhodococcus pyridinivorans* (K408 e AK37), sendo confirmada por testes de alimentação em ratos (KRISZT et al., 2012a; 2012b).

A eubactéria isolada a partir do rúmen bovino é capaz de realizar a degradação do grupo epóxi nas posições C-12, 13 dos tricotecenos, resultando na perda da sua toxicidade. Esta é utilizada como aditivo proposto para limitar o efeito deletério dos tricotecenos (BINDER et al., 2000). Uma das estratégias para utilizar o tratamento biológico, é identificar o micro-organismo que já é capaz de degradar a micotoxina, conhecer e estudar as melhores condições para o seu crescimento no trato gastrointestinal e o seu mecanismo de ação. Protozoários do rúmen são reconhecidos por degradar as micotoxinas, mas se os animais forem alimentados com dietas ricas em carboidratos fermentáveis, a sua população é capaz de desaparecer totalmente (KIESSLING et al., 1984; USHIDA; JOUANY; DEMEYER, 1991; WESTLAKE; MACKIE; DUTTON, 1989).

Diante do exposto, alguns critérios devem ser seguidos para o estudo deste tratamento. A resistência do micro-organismo frente a micotoxina precisa ser investigada, assim como, sua diferença de sensibilidade e seletividade (MADHYASTHA et al., 1994). Alguns micro-organismos são capazes de destruir somente algumas micotoxinas, sendo que outras são resistentes ao processo de degradação. Além disso, propriedades organolépticas e nutritivas do alimento, assim como viabilidade econômica adequada devem ser consideradas como parte da pesquisa e de potenciais aplicações.

O efeito das substâncias naturais também são frequentemente investigadas sobre o crescimento fúngico e a produção das micotoxinas nos alimentos (ESPITIA et al., 2012; KOMALA et al., 2012; MORAIS et al., 2008). Compostos derivados de plantas aromáticas e óleos essenciais possuem uma vasta gama de atividades, especialmente atividade antimicrobiana (BURT, 2004). Os óleos essenciais foram classificados como seguros para uso em alimentos (BURT, 2004) e têm sido recomendados como conservantes para produtos alimentares com base em seus efeitos antimicrobianos e anti-micotoxigênicos (TATSADJIEU et al., 2009). Em estudo com extrato de Juca (*Libidibia ferrea* Mart), planta da floresta Amazônica, houve inibição total de

aflatoxinas produzidas por *A. parasiticus* nas concentrações de 1,62 e 3,24% (MARTINS et al., 2014), demonstrando eficiência *in vitro* frente ao fungo testado.

3.5.3 Descontaminação química

Os métodos químicos são aqueles que envolvem reações químicas ou processos químicos para a inativação das micotoxinas, normalmente esses processos se referem à adição de substâncias químicas que ao entrarem em contato com a micotoxina proporcionam uma mudança na conformação da molécula, ou uma ligação. Estes métodos são os mais utilizados na indústria de alimentos, por serem mais rápidos e com custos mais baixos. Podem ser realizados por tratamento com atmosfera modificada (ozônio-O_3, nitrogênio-N_2, gás carbônico-CO_2) e por meio de produtos químicos (compostos orgânicos e inorgânicos).

O tratamento químico de grãos contaminados por processo de amonização utilizando hidróxido de sódio ou amônia gasosa é reconhecida como um procedimento eficiente para reduzir micotoxinas. Quando aplicada no milho contaminado com fumonisinas, durante 4 dias à 50 ºC e pressão atmosférica, a redução da micotoxina foi de 30 à 45% (NORRED et al., 1991). A combinação do tratamento térmico com $NaHCO_3$ e H_2O_2, sozinho ou com $Ca(OH)_2$ reduziu 100% a FB_1 em milho contaminado (PARK et al., 1996). Zhang et al. (2007a) mostrou que o salicilato de sódio, o ácido isonicotínico e o ácido DL-b-amino-n-butírico pode reduzir a severidade da doença da giberela sobre o trigo em até 57% em estudo realizado em casa de vegetação. Um outro composto químico que demonstrou ter eficiência sobre a doença da giberela no trigo e na cevada, foi a quitosana. Este foi capaz de reduzir em até 74% os sintomas da doença na planta em condições de crescimento no campo (KHAN; DOOHAN, 2009). Tratamentos químicos com ácido clorídrico, peróxido de hidrogênio, hipoclorito de sódio, ácido ascórbico e carbonato de amônio também são estudados. Os adsorventes também são alternativas para reduzir a contaminação por micotoxinas. São utilizadas em ração animal para prevenir e tratar micotoxicoses em animais. Estes adsorventes (zeolitas, bentonitas, argilas) podem se ligar à micotoxina, impedindo que sejam absorvidas pelo trato gastrointestinal do animal, sendo excretada pelas fezes (HUWIG et al., 2001).

Os fungos também são controlados pela aplicação de agrotóxicos tanto no campo quanto no armazenamento. Estudos têm

investigado a eficiência de inseticidas em combinação com fungicidas (DE CURTIS et al., 2011; FOLCHER et al., 2009) e apresentam resultados significantes quanto a redução de fungos e micotoxinas do grupo dos tricotecenos. Entretanto, este tratamento tem desvantagens devido a alta toxicidade aos mamíferos e a permanência de resíduos nos alimentos. Por esta razão, o interesse de compostos inorgânicos, tais como o zinco, está aumentando por não serem tóxicos em quantidades adequadas e apresentar forte atividade antimicrobiana em baixas concentrações (BRAYNER et al., 2006; BURGUERA-PASCU; GUANGJIAN et al., 2012; KUMAR et al., 2013; RODRÍGUEZ-ARCHILLA; BACA et al., 2007; SHENG; NGUYEN; MARQUIS, 2005). Além disso, são elementos essenciais para o corpo humano (PRASAD et al., 1995) e podem ser utilizados como fortificante de alimentos. Algumas formas de zinco, como acetato, cloreto, citrato, gluconato, lactato, óxido, carbonato e sulfato são considerados como seguros para uso em alimentos (GRAS) e autorizados para fortificação de alimentos (FDA, 2011; ODS, 2014). Mesmo apresentando estas vantagens, poucos são os estudos que comprovam seu mecanismo de ação frente a fungos toxigênicos (HE et al., 2011; SAWAI; YOSHIKAWA, 2004) e por isso, merecem especial atenção como uma forma alternativa para descontaminação destes micro-organismos.

A ozonização também pode ser proposta como um método de descontaminação química para grãos contaminados com micotoxinas. Este gás é um oxidante poderoso reconhecido desde 1997 como uma substância GRAS e aplicado na indústria de alimentos para a destruição ou a desintoxicação de substâncias químicas ou micro-organismos. O O_3 atua mediante a oxidação progressiva de componentes celulares para destruir os micro-organismos, impedindo seu crescimento e a formação da micotoxina (GUZEL-SEYDIM; GREENE; SEYDIM, 2004). Para utilizar métodos químicos como descontaminantes de fungos, micotoxinas e resíduos de agrotóxicos, é importante o conhecimento das suas vantagens e desvantagens, assim como dos critérios básicos dos descontaminantes em alimentos.

3.5.3.1 Nanopartículas metálicas: alternativa para descontaminação química de fungos toxigênicos

A dificuldade de controlar o crescimento de fungos não provém somente das condições ambientais desfavoráveis no campo, mas também do desenvolvimento da resistência dos fungos frente a fungicidas convencionalmente utilizados. Para suprir a necessidade de

reduzir e/ou inibir o desenvolvimento de fungos, é essencial os estudos de novos agentes anti-fúngicos que possam auxiliar nas estratégias atuais de controle. Estes agentes devem causar danos para as membranas fúngicas, entretanto, precisam manter a integridade dos grãos. Os agrotóxicos tradicionalmente utilizados como fungicidas tem várias desvantagens, incluindo toxicidade em altas concentrações e permanência de resíduos nos alimentos (BARLOW, 1985; BOOBIS et al., 2008). Por estas razões, o interesse em compostos inorgânicos, como os óxidos de metais, está aumentando (SAWAI, 2003; SEVEN et al., 2004; YAMAMOTO, 2001; ZHANG et al., 2007b). Alguns metais podem apresentar forte atividade antimicrobiana em baixas concentrações (BRAYNER et al., 2006), são elementos essenciais ao organismo e não são tóxicos se consumidos na quantidade adequada (PRASAD, 1995).

Alguns estudos já foram apresentados utilizando estes compostos com atividade antibacteriana (LIU et al., 2009; SAWAI, 2003; STOIMENOV et al., 2002; ZHANG et al., 2007b; YAMAMOTO, 2001), entretanto, poucos são realizados contra fungos filamentosos (HE et al., 2011; SAWAI; YOSHIKAWA, 2004; SEVEN et al., 2004).

Nos últimos anos, as nanopartículas (NPs) também têm recebido especial atenção quanto as suas propriedades físicas e químicas, especialmente no desenvolvimento de novos produtos com atividade antimicrobiana. A área de nanotecnologia vem crescendo com muitas aplicações na ciência e tecnologia, especialmente, com a finalidade de fabricação de novos materiais em escala nanométrica (ALBRECHT; EVANS; RASTON, 2006). “Nano” é uma palavra grega que significa extremamente pequeno, usada para indicar um bilionésimo de um metro, sendo as NPs aglomerados de átomos na faixa de tamanho de 1-100 nm (NSTC, 2007). As nanopartículas de metais têm uma elevada área superficial específica e uma grande fração de átomos da superfície que tem sido extensivamente estudada devido a suas características físico-químicas únicas, incluindo atividade catalítica, antimicrobiana e propriedades ópticas, eletrônicas e magnéticas (DURAN et al., 2005; KOWSHIK et al., 2003). A nanotecnologia tem se beneficiado da área de segurança alimentar principalmente por meio do desenvolvimento de biossensores altamente sensíveis para detecção de patógenos e desenvolvimento de novas soluções antimicrobianas. Mesmo diante de suas vantagens, uma compreensão clara dos possíveis efeitos das nanopartículas na saúde ainda não está disponível, resultando em uma limitação ao seu uso generalizado, especialmente na área de segurança alimentar (BOWMEESTER et al., 2009; FDA, 2012).

Recentes estudos tem demonstrado atividade antimicrobiana de várias NPs, incluindo a prata (CHOI et al., 2008) cobre (CIOFFI et al., 2005) e óxido de zinco (LIU et al., 2009). A atividade anti-fúngica de NPs de óxido de zinco já foi evidenciada, inibindo o crescimento de *Botrytis cinerea* e *Penicillium expansum* (HE et al., 2011). A atividade antimicrobiana pode ser atribuída à forte citotoxicidade, pois interage com grupos funcionais da superfície da célula microbiana, inativando-a (FENG et al., 2000; YOUNG; LEINWEBER; THOMAS, 2005).

3.5.3.2 Compostos de Zinco: alternativa para descontaminação química de fungos toxigênicos

Zinco é um mineral essencial para o organismo humano e animal, pois atua em processos metabólicos de tecidos e órgãos, especialmente do sistema imunológico. A nutrição com adequada quantidade de zinco é essencial para a saúde humana porque este elemento tem múltiplas funções enzimáticas relacionadas ao metabolismo de proteína, carboidratos, lipídeos e álcool (HESS et al., 2009). Alguns compostos de zinco podem ser utilizados em suplementes alimentares, sendo que as formas de acetato, cloreto, citrato, gluconato, lactato, óxido, carbonato e sulfato são considerados seguros pela GRAS para a fortificação de alimentos (FDA, 2011; ODS, 2014). Eles podem ser utilizados na União Européia para o mesmo propósito (EU, 2002). A quantidade recomendada para ingestão de zinco para adultos, homens e mulheres, é de 11 e 8 mg por dia, respectivamente. Entretanto, os níveis de ingestão toleráveis para adultos é de 40 mg por dia (FDA, 2011; ODS, 2014). Para FAO/WHO (2002), o limite tolerável é de 45 mg por dia.

Estudos tem sido realizados com o objetivo de utilizar o zinco como fortificante nos alimentos (BAUTISTA-GALLEGO et al. 2013; TRIPATHI et al. 2010). Isto porque, o processamento dos alimentos, como o trigo por exemplo, reduz substancialmente a concentração de zinco e também de outros sais minerais, o que aumenta ainda mais a deficiência de zinco na alimentação (KUTMAN et al., 2011; ZHANG et al., 2010). A aplicação de zinco nas plantas de trigo durante seu desenvolvimento no campo, tem sido conduzido para aumentar a quantidade de zinco nos grãos (GHASEMI et al., 2013; KHOSHGOFTARMANESH et al., 2013). Em adição, vale ressaltar que é um micronutriente essencial para plantas, o qual é recomendado como fertilizante para as culturas.

Mesmo apresentando muitas vantagens, poucos são os estudos que reportam a atividade antifúngica dos compostos de zinco (HE et al., 2011; SAWAI; YOSHIKAWA, 2004). O mecanismo sugerido para a atividade antimicrobiana destes compostos pode estar baseado na formação de EROs, que causam alterações na integridade da membrana celular do micro-organismo, levando a danos e também a morte celular (APPLEROT et al., 2009; FENG et al., 2000; HE et al., 2011; LIU et al., 2009).

Recentes estudos na literatura tem demonstrado atividade de compostos de zinco, incluindo nanopartículas de zinco. Sharma e colaboradores (2011) encontraram forte atividade antifúngica de nanopartículas de óxido de zinco frente ao fungo *Pythium debarynum* na concentração de 10 mM. Poucos são os estudos que demonstram atividade antifúngica contra fungos toxigênicos. He et al. (2011) demonstraram que 12 mM de Zn-NPs foram suficientes para inibir completamente o crescimento de *B. cinerea* e *P. expansum*. Kairyte, Kadys, Luksiene (2013) utilizaram Zn-NPs fotoativadas, que foram eficientes para reduzir o crescimento de *Listeria monocytogenes*, *Escherichia coli* e *B. cinerea*. Ainda, ZnO demonstram inibir o crescimento de fungos (*Aspergillus niger* e *Rhizopus stolonifer*) somente em altas concentrações (>100 mg mL^{-1}) por método condutimétrico (SAWAI; YOSHIKAWA, 2004). Propriedades antibacterianas de $ZnSO_4$ também foram estudadas, mostrando que sua adição a ionômero de vidro à base de cimentos levou a inibição significativa do crescimento de *Streptococcus mutans* (OSINAGA et al., 2003). ZnCl demonstrou inibir espécies bacterianas, tais como *Salmonella*, *E. coli*, *Staphylococcus aureus*, *S. hyicus*, *E. faecalis* e *E. faecium* (AARESTRUP; HASMAN, 2004). Em adição, nanoestruturas de ZnO demonstram ter atividade antimicrobiana frente a *S. aureus* e *E. coli* (KUMAR et al., 2013).

Investigar a eficiência da atividade anti-fúngica destes compostos frente a importantes fungos toxigênicos do trigo como os dos gêneros *Fusarium*, *Aspergillus* e *Penicillium*, seria de grande valia para área de tecnologia de alimentos. Destes gêneros, a espécie *F. graminearum* (produtora da doença giberela na planta de trigo) merece especial atenção para o estudo da ação destes descontaminantes.

Seja qual for a estratégia de controle escolhida, o estudo do seu mecanismo de ação frente a fungos toxigênicos torna-se fundamental para a análise de sua eficiência. Estes mecanismos envolvem a estrutura do fungo, formação de conídios, mortalidade das hifas, oxidação das membranas celulares e por fim a formação de micotoxinas. Desta forma,

pode-se avaliar sua funcionalidade e assim propor novas tecnologias e instrumentos que possam impedir o crescimento fúngico e sua toxicidade.

3.5.3.3 Gás ozônio: alternativa para descontaminação química de fungos toxigênicos e micotoxinas

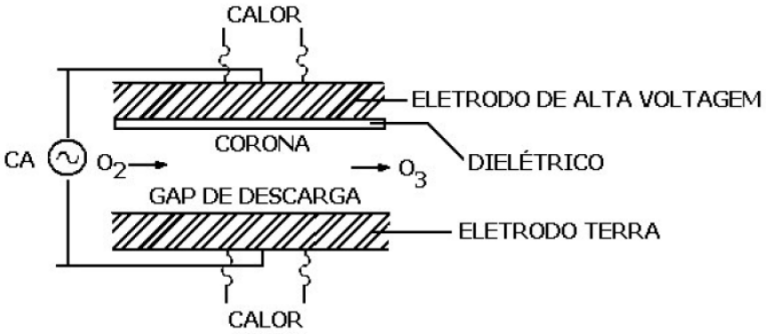
O ozônio é um alótropo triatômico (O_3) composto por três átomos de oxigênio que se formam quando as moléculas de oxigênio se rompem devido a radiação ultravioleta e os átomos separados combinam-se individualmente com outras moléculas de oxigênio. Devido a maior estabilidade do oxigênio, a molécula de O_3 sofre um processo de dissociação espontânea com o tempo resultando novamente na formação do oxigênio (LANGLAIS; RECKHOW; BRINK, 1991). A decomposição não resulta em espécies nocivas já que o mesmo é espontaneamente convertido em O_2. Por ser instável, requer que ele seja produzido no seu local de aplicação reduzindo assim gastos e perigos relacionados como seu transporte e estocagem (ARMOR, 1999; TATAPUDI; FENTON, 1994).

A produção comercial do ozônio é realizada pelo processo de descarga elétrica, também chamado de corona (USEPA, 1999). Este processo é constituído por dois eletrodos submetidos a uma elevada diferença de potencial (aproximadamente 1000 V). O O_3 é gerado pela passagem de ar ou oxigênio puro entre os dois eletrodos. Quando os elétrons possuem energia suficiente para dissociar a molécula de O_2, começam a ocorrer colisões, que causam a dissociação do O_2 e a consequente formação do O_3 (CULLEN et al., 2009; USEPA, 1999) (Figura 7).

É um poderoso agente oxidante e desinfetante (GUZEL-SEYDIM; GREENE; SEYDIM, 2004; KEELS et al., 2001; MCKENZIE et al., 1998; MENDEZ et al., 2003) capaz de participar de um grande número de reações com compostos orgânicos e inorgânicos (ALMEIDA; ASSALIN; ROSA, 2004; KUNZ; PERALTA-ZAMORA, 2002). Pode reagir com a maioria dos compostos contendo ligações duplas, como C=C, C=N, N=N, etc., mas não com grupos funcionais contendo ligações simples, como C-C, C-O, O-H, etc (ALMEIDA; ASSALIN; ROSA, 2004; GOGATE; PANDIT, 2004). O gás O_3 é um poderoso germicida, empregado em engenharia sanitária para a desinfecção da água potável e na remoção de sabores e odores indesejáveis. Também é um gás oxidante, que tem inúmeras aplicações

na indústria alimentícia pelas vantagens que apresenta nas técnicas de preservação.

Figura 7 - Gerador de ozônio tipo descarga corona



Fonte: adaptado de Usepa (1999)

O_3 foi reconhecido como seguro em 1997, sendo que em 26 de junho de 2001, o FDA publicou uma determinação oficial sobre a utilização do O_3 admissível como um agente antimicrobiano para o tratamento, armazenamento e processamento de alimentos em gás e uma fase aquosa em contato direto com os alimentos, incluindo as matérias-primas, além de frutas e hortaliças minimamente processadas (GUZEL-SEYDIM; GREENE; SEYDIM, 2004). O O_3 já se mostrou eficaz contra um amplo espectro de micro-organismos, incluindo bactérias (KIM; YOUSEF, 2000; SHARMA et al., 2002; XU, 1999), fungos (PALOU et al., 2002; PEREZ et al., 1999) vírus e protozoários (CULLEN et al., 2009; KHADRE; YOUSEF; KIM, 2001; RESTAINO et al., 1995). Além disso, também tem potencial para matar pragas de armazenagem (KELLS et al., 2001; MENDEZ et al., 2003), degradar micotoxinas (CULLEN et al., 2009), agrotóxicos e resíduos químicos (HWANG; CASH; ZABIK, 2001; ONG et al., 1996).

Na indústria alimentícia, o tratamento com O_3 vem sendo estudado com o intuito de melhorar a qualidade e evitar perdas quantitativas, devido à deterioração dos alimentos por fungos. O tratamento com gás O_3 tem demonstrado eficiência em reduzir contaminação por AFB_1 em figos secos (ZORLUGENÇ et al., 2008) e PAT em sucos de maça (CATALDO, 2008). Pode desempenhar importante papel na qualidade de castanhas-do-Brasil, já que tem sido efetivo em inibir o crescimento fúngico e reduzir a contaminação por

AFLs (SCUSSEL et al., 2011). Na Tabela 5 estão descritas as aplicações de O_3 para inibição de fungos e degradação de micotoxinas em diferentes alimentos.

A aplicação de O_3 em grãos de trigo pode ser promissor para a qualidade e segurança do alimento, pois além de demonstrar eficiência em reduzir a contaminação por micotoxinas, também apresenta efeitos na remoção de resíduos de agrotóxicos em alimentos. Em estudos recentes, o O_3 já foi utilizado para a remoção de resíduos de agrotóxicos em vegetais e frutas (GABLER et al., 2010; IKEURA; KOBAYASHI; TAMAKI, 2011; IKEURA; HAMASAKI; TAMAKI, 2013; KUSVURAN et al., 2012). Em 2012, Kusvuran et al. avaliaram a eficiência de O_3 aquoso em frutas armazenadas e obtiveram resultados significantes com 5 min de exposição de limão e laranja (armazenadas a 20ºC) em O_3 aquoso na concentração de 10 ppm. Os resíduos de agrotóxicos em limão foram reduzidos em 92, 59,9 e 48,5% para clorotalonil, tetradifon e etil, já para laranja, foram reduzidos em 100, 56,6 e 40,4%, respectivamente.

Tabela 6 - Aplicações de ozônio para inibição de fungos e degradação de micotoxinas em diferentes alimentos

Alimento	Concentração O_3 (ppm)	Tempo de Exposição (min)	Inibição	Referências
Redução de fungos				
Cevada	11 e 26	15	24 e 36% *Fusarium*	KOTTAPALLI; WOLF-HALL; SCHWARZ, 2005
Cevada	0.16	5	96% contagem total	ALLEN; WU; DOAN, 2003
Castanha-do-Brasil	31	300	100% contagem total	GIORDANO et al., 2010
Castanha-do-Brasil	10	90	100% contagem total	SCUSSEL et al., 2011
Figo	13.8	15	100% contagem total	ZORLUGENÇ et al., 2008
Figo	5 e 10	180 e 300	72% contagem total	ÖZTEKIN; ZORLUGENÇ; KIROGLU, 2006
Inativação de micotoxinas				
Amendoim	6	30	65,8 AFLs	CHEN et al., 2014
Amendoim	6	30	65,9 AFB_1	CHEN et al., 2014
Castanha-do-Brasil	31	300	100% AFLs	GIORDANO et al., 2010
Castanha-do-Brasil	10	90	100% AFLs	SCUSSEL et al., 2011
Figo	13.8	30, 60 e 180	48.7; 72.4 e 95.2 AFLs	ZORLUGENÇ et al., 2008
Milho	200	5520	95% AFB_1	MCKENZIE et al., 1998
Milho	47.8	2	30% AFLs	MCDONOUGH et al., 2011
Milho	90	40	88,1% AFB_1	LU et al., 2014
Pimenta vermelha	66	60	93% AFB1	INAN; PALA; DOYMAZ, 2007

Fonte: autor (2014)

Por outro lado, o O_3 gasoso foi eficiente na degradação de agrotóxicos quando aplicado em uvas na concentração de 10 ppm por 60 min, reduzindo 68,5, 75,4 e 83,7% dos fungicidas fenexamida, ciprodinil e pirimetanil (KARACA; WALSE; SMILANICK, 2012).

A ação do O_3 para redução dos compostos químicos, tais como as micotoxinas e os agrotóxicos, pode levar a formação de sub-produtos das reações de degradação, resultante da ozonólise. No entanto, a toxicidade de muitos destes sub-produtos, tem sido reduzida quando comparada ao composto original. O O_3 pode degradar totalmente o composto ou causar modificações químicas, reduzindo sua atividade biológica, isto irá depender da estrutura química a ser degradada (CULLEN et al., 2009; DIAO et al., 2013; MCKENZIE et al., 1997; TIWARI et al., 2010). Nas micotoxinas, tais como AFLs, o O_3 reage com a dupla ligação C8 C9 do anel furano da AFB_1 através do ataque eletrofílico com base no mecanismo de Criegee, e desta forma origina produtos intermediários. A destruição desta dupla ligação do C8 e C9 do anel furano significada que a toxicidade foi reduzida ou mesmo desapareceu (MCKENZIE et al., 1997). Por outro lado, com os agrotóxicos, Chelme-Ayala; El-Din e Smith (2010) avaliaram a degradação com O_3 de dois herbicidas, bromoxinila e trifluralina. Os sub-produtos gerados na degradação de bromoxinila, foram 3,5-dibromo-2,4-dihidroxibenzonitril, 3-bromo-4,5-hidroxibenzonitrile, 3-bromo-4-hidroxibenzonitrile and 4-hidroxibenzonitrile. Para trifluralina, os produtos formados durante a ozonização foram α,α,α-trifluoro-2,6-dinitro-*N*-dipropil-*p*-toluidine, 2,6-dinitro-4-trifluoromethianiline e 4-trifluoromethianiline. No entanto, não foi analisado a toxicidade destes sub-produtos de degradação. Malation, diclorodifeniltricloroetano, diazinon, metil paration e paration também foram avaliados juntamente com os seus sub-produtos originados pelo tratamento com O_3. Os resultados obtidos é que o O_3 pode efetivamente reduzir e degradar os agrotóxicos sem formar sub-produtos tóxicos, especialmente para as comunicações intercelulares funcionais que foram avaliadas nas células de fígados de ratos nestes estudos (MASTEN et al., 2001; WU et al., 2007).

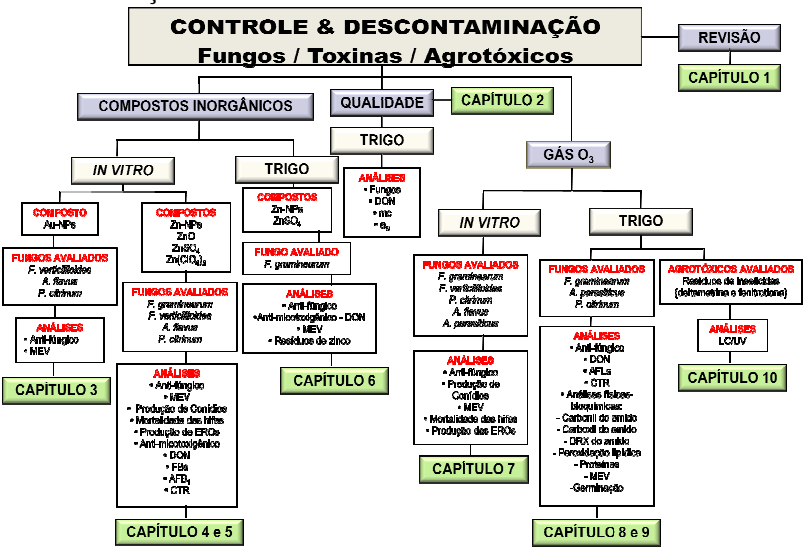
Para o uso seguro do gás O_3 na indústria alimentícia, é necessário conhecer as normas regulamentadoras que tratam dos limites máximos permitidos de concentração e período de exposição ao gás. No Brasil, a Norma Regulamentadora Nº 15, Portaria Nº 3.214/78 estabelece que o limite de exposição ao ozônio pelos trabalhadores seja de 0,08 ppm para uma jornada de trabalho de 48h semanais (BRASIL, 1978). Nos Estados Unidos, os limites permitidos de concentração e

período de exposição ao ozônio é de 0,1 ppm por até 8h por dia (CFR, 1997; FDA, 2005). A temperatura ambiente, o ozônio pode ser perceptível em concentrações de 0,01 a 0,02 ppm, podendo ser detectado pelos trabalhadores antes que uma concentração de risco seja alcançada. Mesmo em concentrações críticas, os efeitos imediatos do O_3 não são tóxicos, apenas sintomáticos. Por isso, em um local de trabalho é importante a instação de um detector para medir a concentração do gás O_3 na faixa de 0,01 a 100 ppm, para conhecer a concentração residual emitida na saída do sistema (KHADRE; YOUSEF; KIM, 2001).

A eficácia do O_3 depende de vários fatores, incluindo a concentração de O_3 aplicada, as características de cada alimento e os fatores ambientais em que se encontram (temperatura e umidade). O excesso do O_3 é auto-decomposto rapidamente produzindo O_2 e assim não resta resíduo no alimento (NAITO; TAKAHARA, 2006). Demonstra ser uma alternativa potencial que oferece vantagens para melhorar a qualidade e a produção dos alimentos.

O Figura 8 representa o fluxograma geral dos estudos de controle e descontaminação, incluindo todos os capítulos deste trabalho apresentados a seguir.

Figura 8 - Fluxograma geral de todo o estudo de controle e descontaminação



Fonte: autor (2014)

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4 CAPÍTULO 2
Micoflora e Deoxinivalenol em Grãos de Trigo (*Triticum aestivum* L.) do Sul do Brasil

ARTIGO PUBLICADO: Savi, Geovana Dagostim, Piacentini, K., Scussel, V.M. Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil. Food Additives & Contaminants 7, p. 232-237, 2014. DOI: 10.1080/19393210.2014.898337

Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil

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Abstract

The fungal species *Fusarium graminearum* is related to deoxynivalenol (DON) formation. The aim of this study was to evaluate mycoflora and DON occurrence in 53 whole wheat grain samples collected in Southern Brazil during the 2012 crop. Wheat grains showed adequate values of water activity ranging from 0.48 to 0.72, within the required limits of moisture content, ranging from 9.1% to 13.9%. In addition, low counts of fungal colonies, ranging from 10 to 8.2×10^2, were found. For *Fusarium* genera, there was predominance of *Fusarium verticillioides* (34%) and *F graminearum* (30.2%). For *Aspergillus* species, 37.7% of *Aspergillus flavus* was determined. Regarding the *Penicillium* species, *Penicillium digitatum* (49%) was the most found species. DON was detected in 47.2% (25 out of 53) of the samples analysed, with levels ranging from 243.7 to 2281.3 µg kg^{-1} (mean: 641.9 µg kg^{-1}).

Keywords: *Fusarium graminearum*; wheat grains; deoxynivalenol; mycoflora

Introduction

Cereals may be contaminated by toxigenic fungal strains under favourable conditions during all stages of plant development in the field and storage steps (Scussel et al. 2011). The main fungal genera reported in harvest or stored grains are *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* (Bräse et al. 2009). However, *Fusarium*, *Aspergillus* and *Penicillium* sp. are more often found in wheat grain worldwide (González et al. 1996, 1998; Berghofer et al. 2003; Muthomi

et al. 2008; Riba et al. 2008; Roigé et al. 2009; Riba et al. 2010). In the literature, Fusarium species, especially *Fusarium graminearum*, is frequently associated with Fusarium head blight (FHB) in wheat (Del Pont et al. 2012). FHB is a fungal disease that occurs primarily by inoculum of *F. graminearum* in the wheat plant. *F. graminearum* appear to be capable of producing ascopores (sexual spores) in the natural state, which are produced in perithecia on wheat crop residues. These spores are carried by wind over long distances and deposited in anthers, cause infection in the plant and, consequently, promote depigmentation of affected spikelets. Depigmentation occurs as green to white/brown/yellow. If the environment is moist enough, pathogens produce masses of conidia, which appear pink in colour. Conidia are also a major source of inoculums (Panisson et al. 2002; Lima 2004).

Among the mycotoxins associated with FHB, such as deoxynivalenol (DON), nivalenol, T-2 and HT-2 toxins, DON is the most common in wheat grains (Muthomi et al. 2008; Bensassi et al. 2010; Soleimany et al. 2012; Stanković et al. 2012; Santos et al. 2013). The accumulation of DON in human and animal bodies after ingestion of contaminated food can induce acute and chronic effects, such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity (Rotter et al. 1996; Wijnands & Van Leusden 2000; Pestka 2007).

In the last two years in Brazil, around 5.09 million tons of wheat were harvested, mainly in the Southern Brazil region (94%). Nevertheless, the annual national demand for wheat grain is about 10 million tons. For this reason, Brazil imports approximately 5.7 million tons of wheat grain, mainly from Argentina (CONAB 2012). The sub-tropical climate in this region may cause exposure of grains and seeds to contamination by fungi and mycotox-ins, due to environmental factors, mainly temperature and humidity (Astolfi et al. 2012; CONAB 2012).

Since 2012, the Brazilian regulation has proposed maximum levels (MLs) of 2000 µg kg^{-1} for DON in whole wheat grains for human consumption. This limit will decrease over time to allow grain producers and the industry to meet the legislation requirements, without causing a shortage of wheat. From January 2014, DON limits for whole wheat grain will be set at 1500 µg kg^{-1} and in January 2016, at 1000 µg kg^{-1} (Brasil 2011). Nowadays, the lower limit of DON is equal to 1250 µg kg^{-1}, as fixed by the European Commission for unprocessed cereals and 1750 µg kg^{-1} for unprocessed durum wheat (EC 2006a).

In order to evaluate whether the quality of wheat produced in Brazil is in accordance with the Brazilian regulation, this paper aims to assess the occurrence of DON contamination in whole wheat grains,

especially in the most important productive regions in Southern Brazil. In addition, the detection and identification of mycoflora in wheat grains was performed. A method was validated for determination of DON content in whole wheat grains, involving mycotoxin extraction from the sample by a clean-up step using an immunoaffinity column and quantitative analysis by high-performance liquid chromatography (HPLC)/UV.

Materials and methods

Reagents and materials

Culture media and reagents used were Pro-analysis grade. Potato dextrose agar (PDA), malt extract agar (MEA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil). Czapek-dox, 25% glycerol nitrate (GN25), czapek yeast extract (CYA) media and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil).

DON standard was supplied by Sigma Aldrich Chemicals (St. Louis, MO, USA), reconstituted in a con-centration of 1 mg ml^{-1} acetonitrile and stored at –20°C before use. Working standard solutions, ranging from 0.15 to 15 µg ml^{-1}, were prepared from suitable dilutions of the stock solution in the mobile phase (acetonitrile:water, 10:90, v/v) and stored at 4°C. The solvents acetonitrile and methanol were obtained from Vetec (Duque de Caxias, RJ, Brazil) at LC grade. High-purity milli-Q water (18.2 MΩ cm^{-1}) was obtained from a Millipore Synergy system (Millipore, Bedford, MA, USA). For sample clean-up step, an immunoaffinity column from DON-Test (Vicam, Milford, MA, USA) was used according to the manufacturer procedures.

Instruments

Moisture content (mc) and water activity (a_w) were determined using a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil) and Aqua-Lab 4TE Decagon Devices (Sao Jose dos Campos, SP, Brazil), respectively.

Regarding the mycological tests, the following equip-ment were required: light microscopes (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil);

rotary shaker, Marconi (Piracicaba, SP, Brazil) and microbiological incubator, Quimis (Diadema, SP, Brazil).

Whole wheat samples were ground in a laboratory mill Romer 1301 (Union, MO, USA). The determination of DON was carried out by HPLC equipment model 321 of Gilson (Middleton, WI, USA) equipped with an isocratic pump model 805, a manual injector (20 μL loop), na ultraviolet–visible (UV) detector model 118 set at 218 nm and a chromatographic column C18 250 × 4.60 mm reversed-phase, with 4 μ particle size Fusion-RP 80, Phenomenex (Madrid Avenue, Torrance, CA, USA).

Sampling

A total of 53 whole wheat grain samples were collected during the 2012 crop, of different varieties recommended for cultivation in Rio Grande do Sul-RS, Parana-PR and Santa Catarina-SC states, in Southern Brazil. The samples were collected from bulk batches, after dirt removal and drying (up to a maximum of 60°C) in the storage units. Collection was performed using a grain auger from differ-ent points of the bulk batches, with a minimum final weight of ca. 10 kg. Each sample was homogenised and reduced in portions varying around 2.0 kg. Samples were packed in polyethylene bags and stored at 4°C for immediate analysis of mycoflora, in the Laboratory of Mycology, and DON analysis, in the Laboratory of Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences at the Federal University of Santa Catarina, Florianopolis, Santa Catarina.

Moisture content, water activity and mycological analysis

To determine mc, wheat grains (2 g) were submitted to a drying process in an oven (105 ± 5°C) up to a constant weight using gravimetric method. In addition, for a_w determination, the wheat grains (2 g) were submitted to Aqua-Lab 4TE equipment. All analyses were performed in triplicate and in accordance to Association of Official Analytical Chemists – AOAC (2005).

Mycological analysis was applied as enumeration technique to evaluate fungal total load (Silva et al. 2010). Twenty-five grams of each sample was added to 225 ml of 0.1% peptone dissolved in water in sterile conditions. The mixture was stirred on a rotary shaker for 2 min., dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained, 0.1 ml aliquots of

each dilution were spread (in duplicate) on the surface of the PDA medium containing chloramphenicol (100 mg l^{-1}) and incubated for a max-imum of 7 days, at 28°C in the dark.

The results were presented taking into account the colony forming units per gram (CFU g^{-1}) in the dilution 10^{-1}. For identification of fungal genera and species, the isolated strains were sub-cultured on PDA, MEA, GN25 and CYA media. Species identification was performed through microcultivation in carnation leaf agar for *Fusarium* and Czapek-dox for *Aspergillus* and *Penicillium* species, as described by Weber and Pitt (2000) and Samson et al. (2006). The isolates were examined under a light microscope (100× and 400× magnifications) and spe-cies identification was carried out according to available taxonomic keys and guides (Raper & Fennel 1965; Pitt 1979; Nelson et al. 1983; Pitt & Hocking 1997).

DON quantification

Whole wheat samples (2.0 kg) were ground in a Romer mill (with automatic quartering) and portions of 25 g were taken for DON analysis, using clean-up with immunoaffinity columns according to the Vicam protocol DON Test, No. G1005 USA (Vicam 2013) with slight modifications. Briefly, 25 g of each sample were mixed into an industrial blender and set into an industrial blender jar with 100 ml of ultra pure water. The mixture was blended for 30 seconds, filtered twice and cleaned by an immunoaffinity column (DONTest HPLC), which was first conditioned with 1 ml ultra pure water, at a flow rate of one drop per second. After washing the column with 2.5 ml of ultra pure water, the mycotoxin was slowly eluted with 2 ml of 100% LC grade methanol. The eluate was evaporated using a heating block set at 40°C with a gentle nitrogen stream and the dry residue was then redissolved in 100 µL of mobile phase (acetonitrile:water, 10:90, v/v). The extract (20 µL) was injected in the LC/UV system set at 218 nm and a flow rate of 0.6 ml min^{-1}. The DON quantification was per-formed by measurement of peak area at DON retention time compared with the standard solutions used for the calibration curve (0.15, 0.20, 0.25, 0.50, 1, 2, 3, 4, 5, 7.5, 10 and 15 µg ml^{-1}) with correlation coefficient (r) = 0.996. Recoveries were obtained by spiking DON-free wheat samples at DON concentrations of 250, 1000 and 1500 µg kg^{-1} at the same day and under same HPLC conditions. This work was performed in the LABMICO laboratory, which is accredited by Ministry of Agriculture and Food Supplies (MAPA), following ISO/IEC 17025 (2005)

procedures. For quality control, samples were analysed on five different days. Measurement uncertainty (data not shown in the table) was performed according to European Commission Regulation No. 401/2006 (EC 2006b).

Results and discussion

Moisture content, water activity and mycological analysis

Under favourable conditions, such as high temperature (25–35 °C), mc (13–16%) and a_w (0.70–0.90), toxigenic fungi can produce mycotoxins (Scussel 2002). Therefore, maximum mc required in wheat, according to official Brazilian classification, is 13%, as defined in Normative Instruction No. 7 (Brasil 2010). In this study, the whole wheat grains showed values from 0.48 to 0.72 (mean 0.58 ± 0.01) a_w, which meet the required mc limits ranging from 9.14% to 13.94% (mean: 11.38 ± 1.06%). In addition, it presented low count fungal colonies, ranging from 10 to 8.2×10^2 (mean: $1.9 \times 10^2 \pm 1.4 \times 10^2$ CFU g^{-1}). According to the Brazilian regulation, there is no maximum limit for fungal amount in unprocessed wheat grains; however, the presence of toxigenic fungal species in food can be indicative of potential mycotoxin accumulation.

Table 1. Fungal species isolated from whole wheat grain samples from regions in South Brazil.

Wheat grains		Total samples contaminated	Isolated fungi (%)[a]
Genera	Species		
Fusarium			
Fusarium	*F. verticillioides*	18	34.0
	F. graminearum	16	30.2
	F. oxysporum	1	1.9
Other fungi			
Aspergillus	*A. flavus*	20	37.7
	A. parasiticus	7	13.2
	A. nivea	6	11.3
	A. penicillioides	2	3.8
	A. oryzae	2	3.8
	A. terreus	2	3.8
	A. niger	1	1.9
	A. ochraceus	1	1.9
Penicilium	*P. digitatum*	26	49.0
	P. olsonii	5	9.4
	P. expansum	4	7.5
	P. thomii	4	7.5
	P. citrinum	2	3.8
Mucor	*M. plumbeus*	9	17
Cladosporium	*C. herbarum*	4	7.5
Trichoderma	*T. harzianum Rifae*	3	5.7
Cunninghamella	*C. bertholettia*	3	5.7
Rhizopus	*R. oryzae*	3	5.7
Alternaria	*A. alternate*	2	3.8
Byssochlamys	*B. fulva*	1	1.9
Monascus	*M. ruber*	1	1.9

Note: [a]Percentage of isolated fungal species found in the investigated wheat samples (n = 53).

With respect to the whole wheat grain samples, it is possible to highlight the presence of different isolated fungal genera, especially the *Fusarium* (34% of *Fusarium verticillioides* and 30.2% of *F. graminearum*), *Aspergillus* and *Penicillium* spp., as shown in Table 1. *Fusarium graminearum* can be responsible for DON production, as found in this study. DON levels in wheat grains are determined by conditions in the field before harvest. In Southern Brazil, rain and high humidity periods in the flowering periods can increase contamination by *F. graminearum*, which can cause FHB and DON accumulation. It is important to mention that the level of resistance of wheat cultivar also has effects on DON contamination (Astolfi et al. 2012; Del Pont et al. 2012). Previous studies in the country showed DON to be the main mycotoxin in wheat grains (Calori-Domingues et al. 2007; Santos et al. 2013). Similarly, in wheat grains collected in Argentina, *F. graminearum* also was the most frequently isolated species (González et al. 1996, 1998), like it was in Kenya (Muthomi et al. 2008). Co-occurrence of DON with other mycotoxins in naturally contaminated wheat, such as fumonisins (FBs), ochratoxin and zearalenone, was also reported (Birzele et al. 2000; Marques et al. 2008; Stanković et al.

2012). The major FB-producing fungus, *F. verticillioides*, was often found in this study.

Among the *Aspergillus* species, the most found was *Aspergillus flavus* (around 37.7%), followed by *Aspergillus parasiticus* (13.2%) and *Aspergillus nivea* (11.3%), which is in accordance with the Riba et al. (2008, 2010) studies. *Penicillium* genera were often found in this study, with predominance of *Penicillium digitatum* (49%) and *Penicillium olsonii* (9.4%). These genera are also men-tioned in studies in Argentina (Roigé et al. 2009) and Australia (Berghofer et al. 2003). Other fungal species were found in lower predominance (Table 1).

Method validation of DON

The HPLC/UV method for DON separation and the valida-tion parameter linearity, limits of detection and quantification (LOD and LOQ), reproducibility, repeatability and recovery obtained have shown to be quite adequate. Under the chromatographic conditions used, the retention time (Rt) of DON was ca. 17 ± 0.5 min. Linearity was determined from the calibration curve, which was linear in the range 0.15–15 µg ml^{-1}, with a correlation coefficient (r) equal to 0.996. The LOD (signal-to-noise ratio = 3) and LOQ (signal-to-noise ratio = 10) were 66.7 and 119.1 µg kg^{-1}, respec-tively. The recovery experiments were determined by blank wheat grains spiked with DON at concentrations of 250, 1000 and 1500 µg kg^{-1} and analysed in triplicate. They showed yields equal to 87 ± 9%, 96 ± 6% and 93 ± 3%, respectively, with a mean of 92 ± 4%.

DON levels in wheat grains

DON was detected in approximately 47.2% (25 out of 53) of the samples analysed, at levels of 243.7 to 2281.3 µg kg^{-1} (mean: 641.9 µg kg^{-1}), as presented in Table 2. Considering European Comission rules (2006a) regarding to the ML for DON in unprocessed cereals (set at 1250 µg kg^{-1}) and the Brazilian regulations (set at 2000 µg kg^{-1}), only 4 (7.5%) and 1 (1.9%) samples disagreed in both regulations, respectively. However, considering future Brazilian regulations for DON levels in whole wheat grains from 2016, foreseen at 1000 µg kg^{-1}, four (7.5%) samples would be in regula-tion disagreement (Brasil 2011). Similar to these results, Santos et al. (2013) found in northern and central/south-western region of Parana state, Brazil, 66.4%

contaminated samples with DON levels from 206.3 to 4732.3 µg kg^{-1} (mean 1894.9 µg kg^{-1}). In 2007, Calori-Domingues et al. reported for Southern states of Brazil in Sao Paulo, Parana and Rio Grande, 94% DON-contaminated wheat grains with levels from 90 to 4573 µg kg^{-1} (mean 332 µg kg^{-1}). Other studies reported DON occurrence in wheat grains worldwide, as presented in Table 3.

Table 2. DON levels in whole wheat grain samples from regions in South Brazil.

Number of wheat samples	Distribution levels of DON (µg kg^{-1})	Frequency (%)	Positive samples (µg kg^{-1})	Mean contamination of positive samples (µg kg^{-1})
28	<119.1[a]	52.8	0	0
13	200–500	24.5	243.7	320.9
			257.8	
			258.6	
			287.2	
			287.8	
			291.3	
			298.5	
			318.3	
			358.9	
			362.3	
			374.1	
			405.9	
			427.6	
8	500–1000	15.1	508.8	677.0
			527.3	
			547.3	
			726.2	
			731.5	
			746.5	
			805.2	
			823.65	
3	1000–2000	5.7	1370.1	1392.5
			1382.9	
			1424.6	
1	2000–3000	1.9	2281.3	2281.3
Total: 53		100	243.7–2281.3	641.9

Note: [a]Method LOQ = 119.1 µg kg^{-1}.

Table 3. DON levels in wheat grain samples found in other studies.

Country		Frequency (%)	Range (µg kg^{-1})	Mean (µg kg^{-1})	References
Brazil		94	90–4573	332	Calori-Domingues et al. (2007)
Brazil		66.4	206.3–4732.3	1894.9	Santos et al. (2013)
Serbia	2005[a]	85.7	52–3306	605.5	Stanković et al. (2012)
	2007[a]	93.3	50–1090	282.8	
Kenya	Nakuru	75	105–303	132.7	Muthomi et al. (2008)
	Nyandarua	59	105–289	113	
Tunisia		83	12800–30500 µg kg^{-1}	21520	Bensassi et al. (2010)
Malaysian		25	5.5–18.6	–	Soleimany et al. (2012)
India		40	70–4730	910	Mishra et al. (2013)
Morocco		11.1	321–1310	502.1	Ennouari et al. (2013)

Note: [a]year of the study.

Conclusion

Whole wheat grain samples produced in Rio Grande do Sul-RS, Parana-PR and Santa Catarina-SC states in Southern Brazil, showed low fungal load and DON levels below the ML, as set by the EU and Brazil regulations in almost all samples. High contamination levels occurred in 4 (7.5%) samples above the EU ML, which in only 1 (1.9%) sample exceeded the Brazilian ML (2000 µg kg^{-1}). However, the heterogeneous and wide occurrence of DON, even at low concentrations in some cases, must be a matter of concern and show the importance the study. The knowledge of mycoflora and DON levels may contribute to the adoption of corrective measures, such as prevention and control by means of grain-monitoring programmes throughout the production and storage periods. Thus, it will be possible to verify quickly any irregularity and consequently adopt correc-tive measures to reduce risks of mycotoxin contamination.

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5 CAPÍTULO 3
Atividade Biológica das Nanopartículas de Ouro frente a Fungos Toxigênicos

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Biological Activity of Gold Nanoparticles towards Filamentous Pathogenic Fungi

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Abstract. Gold nanoparticles (GNP) were synthesized, characterized and their antifungal activities investigated against three pathogenic fungi of different genera and species (*Fusarium verticillioides*, *Penicillium citrinum* and *Aspergillus flavus*). The anti-fungi treatments efficiency of the GNP (concentrations: 0, 0.05, 0.1 and 0.2 mg L^{-1} in PDA media) were evaluated at 2, 4, 6 and 8 days after incubation by measuring the diameter of fungal colonies and investigating fungi structure alterations by scanning electron microscopy (SEM). It was observed that the GNP concentration increased, fungal colony growth diameter reduced. However, the highest GNP concentration applied in the experiment was not able to completely inhibit fungal growth. The SEM analysis of the fungi structure Au treated showed damaged hyphae and unusual bulges that were not observed in fungi that growth on medium without treatment (Control). Although up to the highest concentration of GNP media applied did not completely inhibited fungi growth, the hyphae modifications led growth reduction which could influence the toxins production by these fungi.

Key-words: gold nanoparticles, activity antifungal, fungal colony, structure hyphae.

1. Introduction

Environment conditions (temperature and relative umidity) during plant growth are key factors regarding fungi development and mycotoxin production on the field [1,2]. Fungi are largely responsible for plant, grain and food products deterioration either pre- and post-harvest. In the storage they are responsible for grains heating which lead to losses of germination, discoloration, reduction of nutritional values and odor changes. Those alterations can affect the quality and safety of raw and processed grains [3]. The main toxigenic fungi that can grow in the plants/grains on the field are *Fusarium* sp. and during grains storage are *Aspergillus* and *Penicillium*. They can contaminate foods either, raw or processed, especially those containing cereals, pulses and fruits [3,4].

The difficulty on controlling fungi growth comes not only from adverse environmental conditions in the field, but also the development of fungi resistance against fungicides conventionally used. To reduce and/or inhibit fungi growth, the development of new antifungal agents that can assist in the current control strategies is essential.

In recent years, nanoparticles (NPs) have received special attention due to their physical and chemical properties, especially for the development of new products with antimicrobial activity. Studies have demonstrated antibacterial activity of various NPs, including silver [5] copper [6] zinc oxide [7,8] and gold [9]. The antimicrobial activity can be attributed to their strong cytotoxicity and interaction to functional groups of the microbial cell surface, inactivating them [10,11,12]. Despite the advantages of GNP, there are only few studies that report the antibacterial [9,13] and antifungal [14] activity of these compounds.

It is already known the size-dependent toxicity of GNP towards different cell types [15] and that it can occur conjugation of GNP with biological important molecules, such as, oligosachharides, DNA and proteins inside cells [16]. Besides, the gold core is essentially inert, non-toxic [17] and its ease of synthesis is relatively advantageous, provided that mono-disperse NPs are formed with core sizes ranging from 1 to 150 nm [18]. Recent works have suggested that the physical GNP parameters can significantly induce cellular responses even with nonspecific binding or uptake inside the cells [19,20].

In this study, the GNP were synthesized, characterized and their antifungal activities investigated towards three filamentous toxigenic fungi: *Fusarium verticillioides*, *Penicillium citrinum* and *Aspergillus flavus*. In addition, by scanning electron microscopy (SEM) their conidia fungi GNP treated structure alterations registered for the first

time.

2. Materials and Methods

2.1 Synthesis and Characterization of gold nanoparticles

The gold nanoparticles were prepared as described by Turkevich et al. [21]. All glass vials were washed in aqua regia and rinsed in ultrapure water. Briefly, an aqueous solution of sodium citrate was added to a hydrogen tetrachloroaurate ($HAuCl_4$) solution previously heated to 90 ºC. The system was maintained under reflux, with magnetic stirring, for 20 min. All chemicals used were of the highest analytical grade and purchased from Sigma Aldrich (St Louis, MO). The GNP were characterized by ultraviolet-visible spectroscopy (UV-vis) in a Shimadzu spectrophotometer model UV-1800 and the XRD patterns obtained with a model 6000 LAB-X equipment, also from Shimadzu. The microstructural characterization was performed in a field emission scanning electron microscope (SEM-FEG) JEOL JSM-7401F.

2.2 Antifungal test

The antifungal GNP activity evaluation was performed according to the Fraternale et al. [22] method. The fungi strains utilized in the experiment were: *F. verticillioides*, *P. citrinum* and *A. flavus* obtained from the culture collection of the Mycological Laboratory at the Federal University of Santa Catarina. The autoclaved PDA media was purred into the Petri dishes with GNP at different concentrations: 0.05, 0.1, 0.2 mg L^{-1} and a control kept without NPs added. The fungi strains were inoculated after the PDA media solidified. A disc (6 mm) of mycelia material, taken from the edge of 7 day-old fungal cultures, was placed in the center of each Petri dish and incubated at 25 ºC at 8 days. The efficiency of GNP treatment was evaluated at 2, 4, 6 and 8 days after incubation by measuring the fungal colonies diameters and registering the visual morphological changes. A Control Group was also prepared by inoculating the same strains in PDA without GNP. Samples of those colonies were collected next for SEM analysis preparation.

2.3 SEM analysis

The fungi mycelia utilized for the SEM colonies alterations investigation were those that grew on PDA containing the highest GNP

concentration (0.2 mg L^{-1}) and showing clear alterations compared to the Control. PDA mycelia sections were collected from the fungal culture edges and directly submitted to SEM analysis as follows: *(a) fungi strains stubs and Au-coating preparation* – strains were fixed onto stubs (Ø 1.2 mm, height 0.8 mm), placed in the Au coater machine (SCD500) holder, applied vacuum (up to 10^4 mBar) and coated with a 1.40 nm Au layer; *(b) fungi strains mycelia SEM observation* – stubs with Au-coated fungi strains were transferred to SEM equipment (*JEOL JSM-6390LV*), submitted again to vacuum and cells visualized, identified at different magnifications and registered by micrographies (taken at a voltage of 0.5 a 30kV).

2.4 Statistical analysis

The data of colonies fungi growth were analyzed by analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were expressed as mean ± S.D. and the p values < 0.05 were considered statistically significant.

3 Results and Discussion

One of the basic prerequisites for GNP to be used in different areas of applications is that they are non-toxic and biocompatible to both *in vivo* and *in vitro* environments. In recent year the GNP were analyzed for biomedical applications, as cancer therapy [23], besides of a variety of optical and electrical assays as signal amplification in numerous bio-diagnostic devices [24,25], and piezoelectric biosensor for detection of a food-borne pathogen [26].

In our work the GNP were synthesized and characterized to evaluate antifungal activity towards filamentous pathogenic fungi. The Fig. 1 (A) show the surface plasmon resonance absorption (SPR) spectrum band related to the GNP aqueous emulsion, which reveals a single band with maximum absorption at 522 nm that corresponds to the SPR band characteristic of spherical GNP. The Fig. 1(B) shows the XRD spectrum to GNP. The sharp and highest intense peak for 2θ at 38.1^o is in agreement with JCPDS card and confirms the presence of gold. The nanoparticles showed nearly spherical geometry as observed from SEM-FEG images (Fig. 1 C) and mean diameter of 30 nm, calculated from X-ray diffraction spectrum employing the Scherrer`s equation [27].

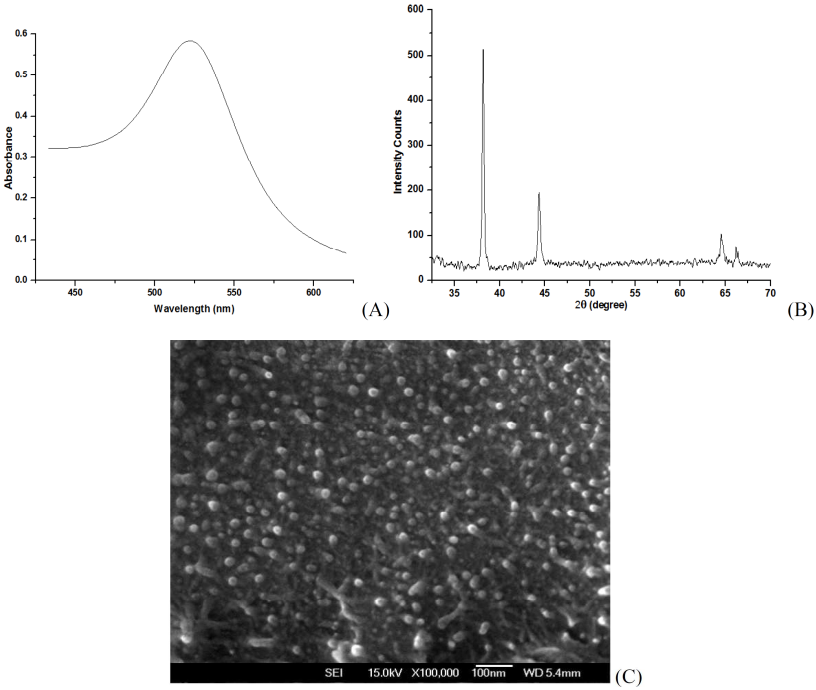

Fig 1. (A) UV-vis spectrum of GNP in aqueous media, (B) X-ray diffraction plot of gold nanoparticles and (C) SEM-FEG micrograph image of gold nanoparticles (x100.000), the sample was covered with Pd.

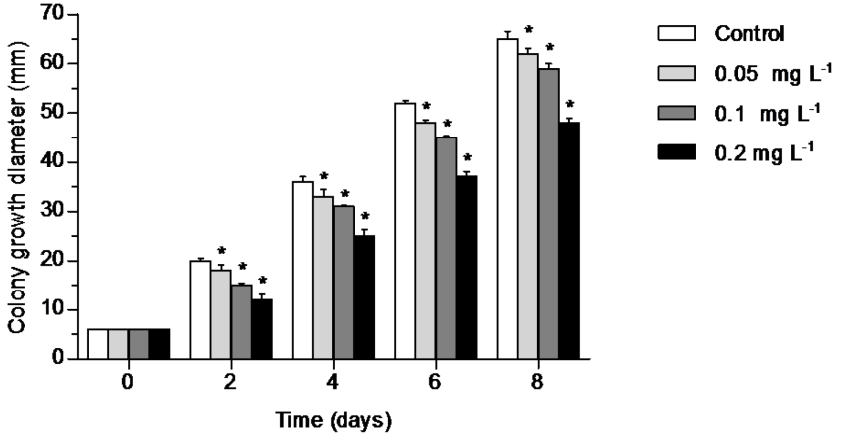

Fig 2. Antifungal activities of GNP against *F. verticillioides* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal colony - each point represents an average of triplicate measurements). Symbols indicate statistically significant when compared with control group *p<0.05.

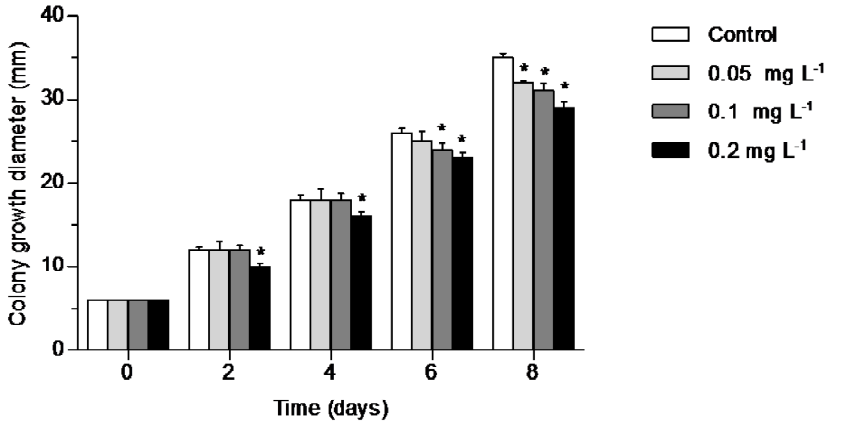

Fig 3. Antifungal activities of GNP against *P. citrinum* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal colony - each point represents an average of triplicate measurements). Symbols indicate statistically significant when compared with control group *p<0.05.

The size and form of the NPs facilitate its passage from one cell to another. This advantage makes the NPs to have different functions within the cell. Studies have shown that GNP can inhibit the growth of bacteria and fungi, by disruption of their cell membrane and change its enzymatic activity. Furthermore, studies have been performed to investigate its synergistic effect with chemicals compounds, as antibiotics. Gu et al. [28] showed that attached vancomycin molecules to the surface of GNP and have greater power to kill than vancomycin on its own, even against vancomycin-resistant bacteria strain. In polysiloxane polymers materials, the presence of GNP enhanced the hydrophobic properties as well as enhancing its bactericidal activity [13]. Currently was verified that GNP exert their antibacterial action against *Escherichia coli*. It can change membrane potential and inhibit ATP synthase activities to decrease the ATP level, indicating a general decline in metabolism; and too to inhibit the subunit of ribosome for tRNA binding, indicating a collapse of biological process. Moreover, the GNP did not induce any reactive oxygen species (ROS) related process, which could explain the low toxicity of GNP to mammalian cells [9].

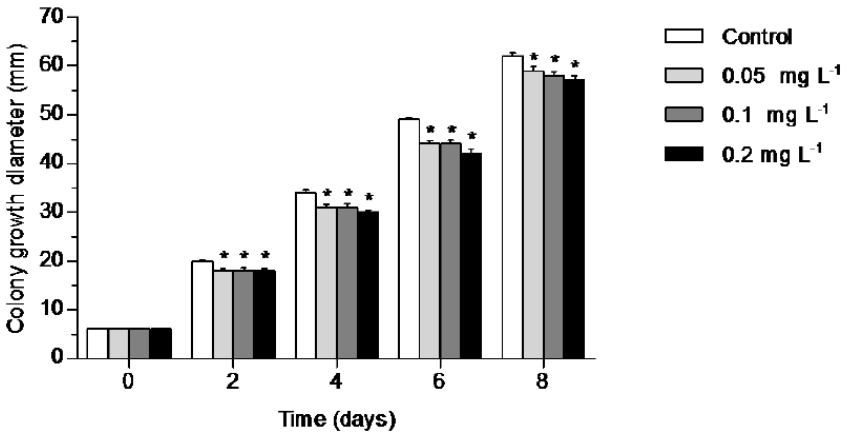

Fig 4. Antifungal activities of GNP against *A. flavus* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal colony - each point represents an average of triplicate measurements). Symbols indicate statistically significant when compared with control group *$p<0.05$.

Regarding fungi, only few studies have reported the antifungal activity and molecular mechanism of action of the GNP. Zhang et al. [14] showed a series of synthesis of GNP prepared successfully by *in situ* reduction and stabilization of hyperbranched poly amidoamine with terminal dimethylamine groups in water. Authors reported GNP antifungal activity against *Aspergillus niger* and *Penicillium* sp. by using the Kirby Bauer modified technique.

In our results, it was verified that by increasing the concentration of GNP, the fungal colony growth diameter reduced. However, the highest concentration used (0.2 mg L^{-1}) was not able to completely inhibit fungal growth when compared to control (without GNP). These results were found either with *F. verticillioides* (Fig. 2), *P. citrinum* (Fig. 3) and *A. flavus* (Fig. 4). *F. verticillioides* and *A. flavus* strains demonstrated better sensitivity when compared to *P. citrinum*, however, all showed significant difference ($p<0.05$) with the NPs treatment in the highest concentration.

The photos of the dish containing culture medium with or without treatment GNP are in the Fig. 5, showing the diameter reduction of the fungal colonies after GNP treatment. The agar dilution method allowed to evaluate the characteristics of fungi studies when grown on culture medium. In the highest concentration used, all the fungi presented colony depigmentation growth in the medium. Therefore, we recon that it could occur any change in the fungus grown exposed to the treatment of NPs.

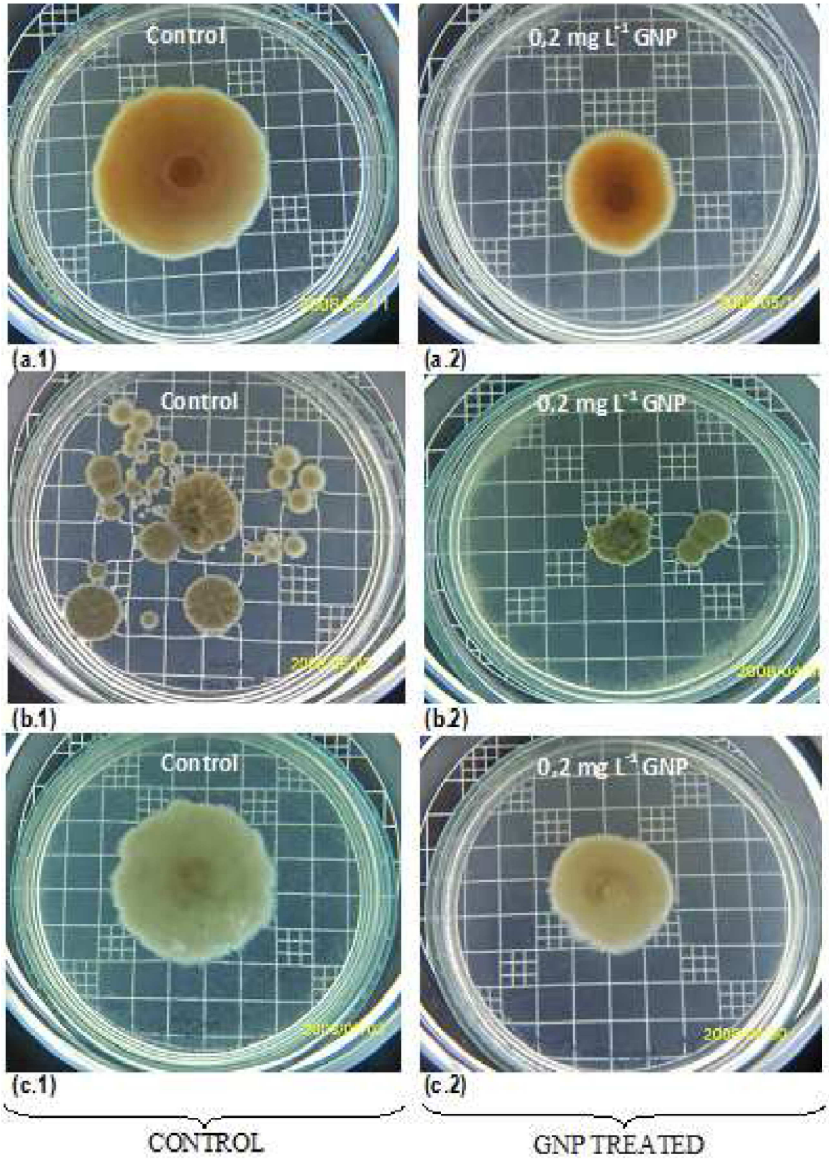

Fig 5. Effect of GNP treatment on the colonies development of (a) *F.verticillioides*, (b) *P. citrinum* and (c) *A. flavus* strains [(1) *Control*: no treatment; (2) *GNP treated*: 0.2 mg L^{-1}].

To investigate if there were changes in the structure of fungi when treated with GNP, the SEM analysis was used. The fungi growth on medium with highest concentration of GNP was analyzed and compared with the control (without GNP treatment). The conidia of the fungi that received treatment showed damage as: deformations, broken and unusual bulges that were not observed in fungi that growth on medium without treatment (control). These results were found with *F. verticillioides* (Fig. 6), *P. citrinum* (Fig. 7) and *A. flavus* (Fig. 8).

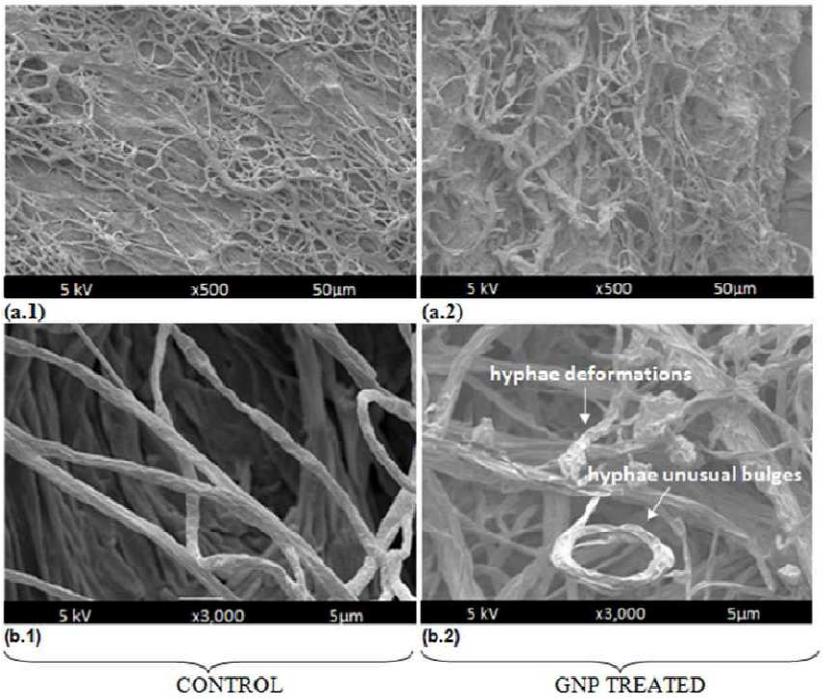

Fig 6. Effect of GNP treatment on *F.verticillioides* mycelia. SEM micrographs images in size of (a) x500, (b) x3,000. [(1) *Control*: no treatment; (2) *GNP treated*: 0.2 mg L^{-1} - showing mycelia alterations].

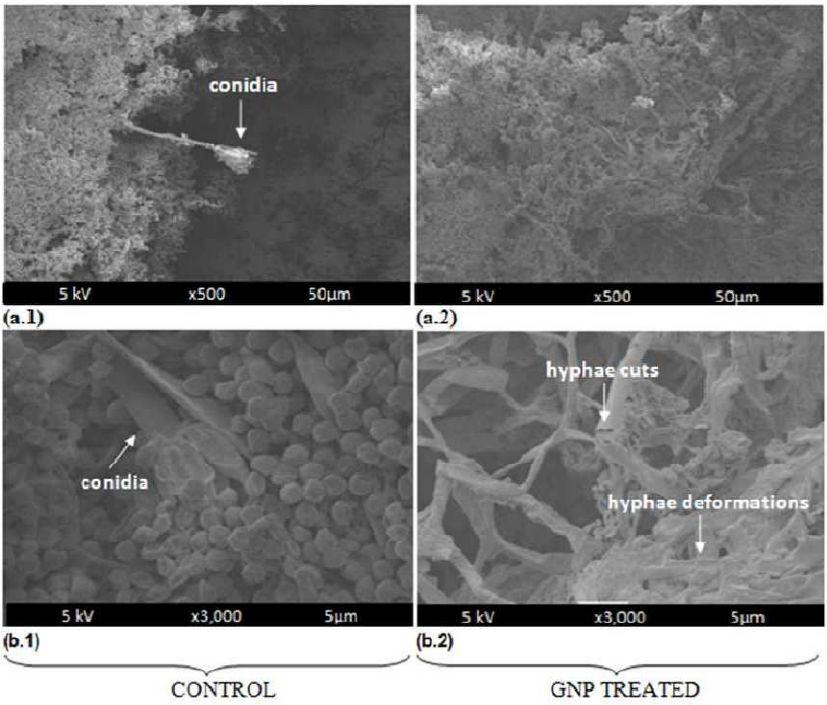

Fig 7. Effect of GNP treatment on *P. citrinum* mycelia. SEM micrographs images in size of (a) x500, (b) x3,000. [(1) *Control*: no treatment; (2) *GNP treated*: 0.2 mg L^{-1} - showing mycelia alterations].

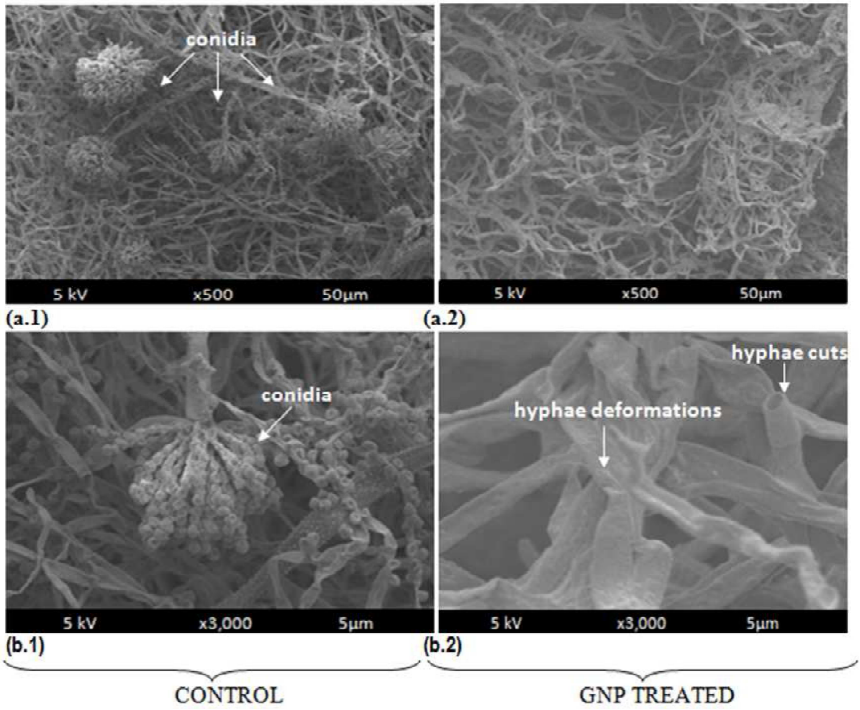

Fig 8. Effect of GNP treatment on *A. flavus* mycelia. SEM micrographs images in size of (a) x500, (b) x3,000. [(1) *Control*: no treatment; (2) *GNP treated*: 0.2 mg L^{-1} - showing mycelia alterations].

These SEM analyzes can confirm the rupture of the fungal cell membrane resulting in possible reduction of the enzymatic activity of the microorganism due to GNP presence. Changes in the structure of the fungus were also observed using zinc oxide nanoparticles against *Penicillium expansum* and *Botrytis cinerea* [7], but this is the first study that demonstrate changes in the structure of the fungus using GNP.

4 Conclusion

Despite the GNP concentration applied in the present study to the toxigenic fungi strains, NPs affected different characteristics of their development.

The highest concentration of GNP utilized in the experiment did not completely inhibit the fungi growth; however it interfered in the

hyphae formation. Fungi hyphae suffered modifications that led to alteration on their growth which were confirmed by SEM observation. Further studies are necessary in order to investigate whether nanoparticles interfere to the cellular fungi metabolism thus inhibiting their secondary metabolites (mycotoxins) production. That will be of great significance for the food safety area, where mycotoxins are one of the main problems regarding contamination.

Acknowledgements

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6 CAPÍTULO 4
Atividade Anti-fúngica de Compostos de Zinco frente a Fungos Toxigênicos e Micotoxinas

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Antifungal properties of Zinc-compounds against toxigenic fungi and mycotoxin

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Summary: Antifungal and antimycotoxin properties of zinc (Zn) compounds were evaluated against toxigenic strains of *Fusarium graminearum*, *Penicillium citrinum* and *Aspergillus flavus*. In addition, was verified the activity of these Zn-compounds on conidia production, hyphae morphological alterations, mortality and reactive oxygen species (ROS) production. The Zn-compounds treatments utilised were zinc oxide nanoparticles (ZnO-NPs), zinc oxide (ZnO), zinc sulphate ($ZnSO_4$) and zinc perchlorate ($Zn(ClO_4)_2$). The Zn-compounds effect on growth diameter of fungal colony was concentration dependent. Two treat-ments ($ZnSO_4$ and $Zn(ClO_4)_2$) completely inhibited the fungal growth and their ability to produce myco-toxins. The conidia production of all fungi also was reduced after the treatment with Zn-compounds. Morphological alterations occurred in the treated fungi showing hyphae damage. The treatments led to cell death and ROS production observed in the fungi hyphae. $ZnSO_4$ and $Zn(ClO_4)_2$ were the compounds that showed better results as antifungal, presenting antimycotoxin activity and caused alterations in the fungi cell structure.

Keywords: Antifungal, antimycotoxin, cell death, ROS production, zinc-compounds.

Introduction

Fungi are responsible for raw and processed grains deterioration when exposed to optimal environment conditions as high temperature and humidity. Some species can cause plant pathologies leading to loss of germination, discoloration and reduction of nutritional values. Moreover, they can produce mycotoxins, especially the *Fusarium* sp. in

crops on the field and *Aspergillus* and *Penicillium* sp. during the storage (Llorens et al., 2004; Scussel et al., 2011).

The main toxins produced by *F. graminearum*, *A. flavus* and *P. citrinum* are deoxynivalenol (DON), aflatoxins (AFLs: AFB_1, AFB_2, AFG_1 e AFG_2) and citrinin (CTR), respectively. DON is responsible for growth rate reduction in animals and humans, anorexia, vomiting, immunotoxicity and brain neurochemical changes; CTR can cause depression, glycosuria, proteinuria and renal lesion; and AFLs are teratogenic, mutagenic and hepatotoxic (Scussel, 1998; Bennett & Klich, 2003). Among the AFLs, AFB_1 presents the highest toxic potential and is classified by the International Agency for Research on Cancer (IARC) as carcinogen, Group 1 (IARC, 1993).

These fungi can be controlled by organic synthetic fungicides application in the field or on storage. However, this treatment has several disadvantages due to high toxicity to mammals and to residuals, which may remain in the foods (Barlow, 1985; Boobis et al., 2008). For these reasons, the interest in inorganic com-pounds, such as zinc (Zn), is increasing (Yamamoto, 2001; Sawai, 2003; Seven et al., 2004; Zhang et al., 2007), because that are nontoxic in appropriates amounts and can present strong antimicrobial activity at low concen-trations (Sheng et al., 2005; Brayner et al., 2006; Burguera-Pascu et al., 2007; Guangjian et al., 2012; Kumar et al., 2013). Moreover, are essential elements for the human body (Prasad, 1995) and also can be utilised as dietary supplements. Some forms as zinc acetate, chlo-ride, citrate, gluconate, lactate, oxide, carbonate and sulphate are considered as generally recognised as safe (GRAS) and authorised for the fortification of foods (FDA 2011; ODS 2011). The recommended amount and the tolerable upper intake levels for Zn depend on life stage. For adults, men and women, the recommended amount is 11 and 8 mg day ^{1}, respectively. However, the tolerable upper intake for adults is up to 40 mg day ^{1} (ODS – Office of Dietary Supplements (National Insti-tute of Health), 2011). Several studies are performed for its utilised as food fortification (Poletti et al., 2004; Akhtar et al., 2008; Tripathi et al., 2010; Bautista-Gallego et al., 2013).

Zn is an essential mineral for physiological and metabolic processes of several tissues and organs, especially to the immune system. In addition, Zn is an essential micronutrient for plants which is recom-mended as fertiliser for crops. Some inorganic Zn-compounds studied are zinc oxide (ZnO), zinc sul-phate ($ZnSO_4$) and zinc perchlorate [$Zn(ClO_4)_2$]. In recent years, zinc oxide nanoparticles (ZnO-NPs) also have received special attention due to their interesting

physical chemical properties and biological application potential as antimicrobial agents (Hanely et al., 2009; Ostrovsky et al., 2009). Nanotechnology has benefited the area of food safety mostly through the development of highly sensitive biosensors for pathogen detec-tion and development of novel antimicrobial solutions. Despite its advantages, a clear understanding of the possible health effects of nanoparticles is still unavail-able resulting in a limitation to its widespread use, especially in the area of food security (Bouwmeester et al., 2009; FDA, 2012).

Zn-compounds are cheap, stable and several studies showed their antibacterial activity, including Zn-NPs (Yamamoto, 2001; Stoimenov et al., 2002; Zhang et al., 2007; Liu et al., 2009). For fungi, there are few studies that report the antifungal activity of Zn-compounds (Sawai & Yoshikawa, 2004; He et al., 2011). The suggested mechanism for the antimicrobial activity of these compounds can be based in the formation of reactive oxygen species (ROS) that disrupt the integrity of the microbial cell membrane, which assists in the damage of microbial enzyme bodies, killing the pathogenic microbe (Feng et al., 2000; Applerot et al., 2009; Liu et al., 2009; He et al., 2011).

Thereby, antifungal properties of Zn-compounds can be studied to assist in the current control strategies against mycotoxigenic fungi. This study aimed to eval-uate the efficiency of Zn-compounds [ZnO-NPs, ZnO, $ZnSO_4$ and $Zn(ClO_4)_2$] on antifungal and antimycotox-in properties against toxigenic strains of *F. graminearum*, *P. citrinum* and *A. flavus*. In addition, the action of these Zn-compounds on conidia production, hyphae morphological alterations, mortality and ROS production was highlighted.

Materials and methods

Zn-compounds

The Zn-compounds, ZnO, $ZnSO_4$ and $Zn(ClO_4)_2$, of analytical grade were purchased from Sigma Aldrich (St Louis, MO, USA).

The ZnO-NPs were synthesised according to Sharma et al. (2011) and characterised by X-ray diffraction (XRD) model Cade-4, Enraf (Nonius-Eugene, OR, USA) and field emission transmission electron microscopy (TEM) model JEM-2100, Jeol (Peabody, MA, USA).

Antifungal and antimycotoxin activities

Antifungal

The fungi utilised in the assays, *F. graminearum*, *A. flavus* and *P. citrinum*, were obtained from the culture collection of the Food Mycology Laboratory of Myco-toxicology and Food Contaminants (LABMICO) at the Federal University of Santa Catarina. The antifungal activity method was performed according to the Fraternale et al. (2003). Different concentrations (10, 25, 50 and 100 mM) of ZnO-NPs, ZnO, $ZnSO_4$ and $Zn(ClO_4)_2$ were added to autoclaved potato dextrose agar (PDA) medium into the Petri dishes, keeping one as Control (PDA without Zn-treatment). A disc (6 mm) of mycelia material, taken from the edge of 7-day-old fungal cultures, was placed in the centre of each Petri dish containing the PDA culture medium Zn-treated and incubated at 25 °C for 8 days. The efficiency of Zn-compounds treatment was evaluated until 8th day after incubation by measuring the fungi colonies diameters.

Antimycotoxin

To evaluate the mycotoxin production, *F. graminearum*, *A. flavus* and *P. citrinum* were grown on PDA medium containing the highest Zn-compounds concen-tration and the Control (PDA without Zn-treatment), at different temperatures (25, 30, 25 °C) and moisture content (80, 90 and 80%) for 20 days, respectively. The fungi grown on each PDA medium was trans-ferred into separating funnel and stirred with chloro-form for mycotoxins extraction, followed by filtration through anhydrous sodium sulphate (Na_2SO_4), which were performed three times. The mycotoxin extract was evaporated with nitrogen stream at 60 °C and quantified by thin layer chromatography (TLC) in plates of silica gel 60 G. The detection of DON, AFB_1 and CTR (produced by the *F. graminearum*, *A. flavus* and *P. citrinum* strains) was performed according to the methods of Trucksess et al., 1984; Moss & Badii, 1982 and Hajjaj et al., 1999, respectively. The extracts were resuspended and the chromatogram developed in the following solvent systems: chloroform:acetone:iso-propyl alcohol (80:10:10), chloroform:acetone (90:10) and chloroform:methanol (50:50), respectively. Standard solutions and the fungi toxin extracts (10 µL) were applied onto silica plates and the chromatographic run performed within 90 min. The plates containing DON and CTR were sprayed with 20% aluminium chloride solution and heated at 120 °C for 7 min to allow mycotoxins

visualisation. Finally, the plates were placed into a 356 nm UV light cabinet to detect the toxins fluorescence. DON and AFB_1 exhibited a bluish and CTR a yellow fluorescence, verified at the following Rfs: 0.63, 0.45, 0.92, respectively.

Conidia production

The *F. graminearum*, *A. flavus* and *P. citrinum* strains were grown on PDA media containing Zn-compounds and control for 8 days at 25 °C. The colonies of fungi were transferred to a 0.89% NaCl and 0.1% Tween 80 solution tube and stirred for fungi conidia detachment. One drop of this conidia suspension was counted in a Neubauer chamber by optical microscope (OM), model CH-Bl 45-2, Olympus (Shinjuku, Tokyo, Japan) in x400 magnification (Marques et al., 2004).

Hyphae morphological alterations, mortality determination and ROS production

Morphological alterations

The treated fungi mycelia sections were collected, fixed with formaldehyde, washed with phosphate buffer solu-tion and dehydrated with alcohol solution (30, 60, 80, 90 and 100%, maintaining the mycelia at 100%) and then submitted to critical point drying according to Bray (2000). After, the fungi mycelia were prepared for scanning electron microscopy (SEM) analysis as follows: fungi strains stubs and gold (Au)-coating preparation – strains were fixed onto stubs (Ø 1.2 mm, height 0.8 mm), placed in the Au Coater, model SCD500, Leica (Leider, IL, EUA) holder, applied vacuum (up to 10^4 mBar) and coated with 1.40 nm Au layer; fungi strains mycelia SEM observation – stubs with Au-coated fungi strains were transferred to SEM microscope model JSM-6390LV, Jeol, submitted again to vacuum, and the cells were visual-ised, identified at different magnifications and registered by micrographies (taken at a voltage of 0.5–30 kV).

Mortality determination

The cell death was verified with Evans blue dye that has the ability to penetrate and remain into nonviable cells. The fungal spores were incubated in saboraud liquid medium for 24 h at 28 °C for hyphae formation and later transferred to this same medium containing the Zn-compounds for another incubation of 24 h at 28 °C. The hyphae were centrifuged, soaked in 0.05% Evans blue solution and left for 5 min.

Finally, the hyphae were washed three times with 1x PBS to remove the excess of dye. The resultant sediment was verified in OM at x400 magnification. The mortality was observed by coloration of blue hyphae (Semighini & Harris, 2010).

ROS production

For the evaluation of the ROS levels in the Zn-treated fungi hyphae, we utilised a 2,7-dichlorofluorescein diacetate (H_2DCFDA) probe that reacts with ROS and becomes fluorescent, thus being possible to evaluate the oxidative stress inside fungi cell structures. The fungal spores were incubated in saboraud liquid med-ium for 24 h at 28 °C, for hyphae formation. After, the hyphae were incubated in this same medium containing Zn-compounds for 24 h at 28 °C. The fungi suspension was centrifuged and treated with 40 µM H_2DCFDA for 30 min at 28 °C in the dark. Finally, the sediments were washed three times with 1x PBS and analysed by confocal optical microscope (COM), model DMI6000B, Leica at 9300 magnification. The ROS production was observed by coloration of green fluorescent hyphae (Liu et al., 2010).

Statistical analysis

The data of colonies fungi growth, antimycotoxin activity and conidia production were analysed by analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were expressed as average values and standard deviation, and the P values <0.05 were considered statistically significant.

Results and discussion

Synthesis and characterisation of ZnO-NPs

ZnO-NPs synthesised and characterised by microwave method, resulted in particle size with mean diameter of 30 nm which is considered and accepted as nanoparti-cles (Sharma et al., 2011). Under the influence of microwave irradiation, in-bulk heating of the materials results in the dipole change of the polar molecules with uniform temperature distribution which lead to molecular agitation and friction breaking them (Mohajerani et al., 2008). Figure S1a shows the XRD pattern of the synthesised ZnO-NPs. All the diffraction peaks are in good agreement with those of hexagonal wurtzite structure of ZnO

(JCPDS card 36-1451) The peaks of sharp intensity between 30° and 40° theta scale can be indexed to the wurtzite ZnO with high crystallinity (Guo et al., 2005). TEM analysis showed in the Fig. S1b ZnO-NPs with nearly spherical geometry and mean diameter of 30 nm (Becheri et al., 2008).

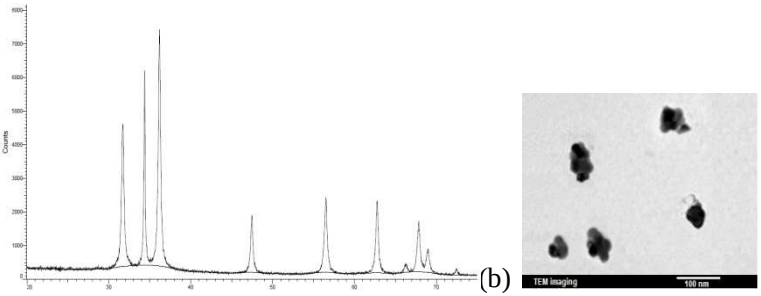


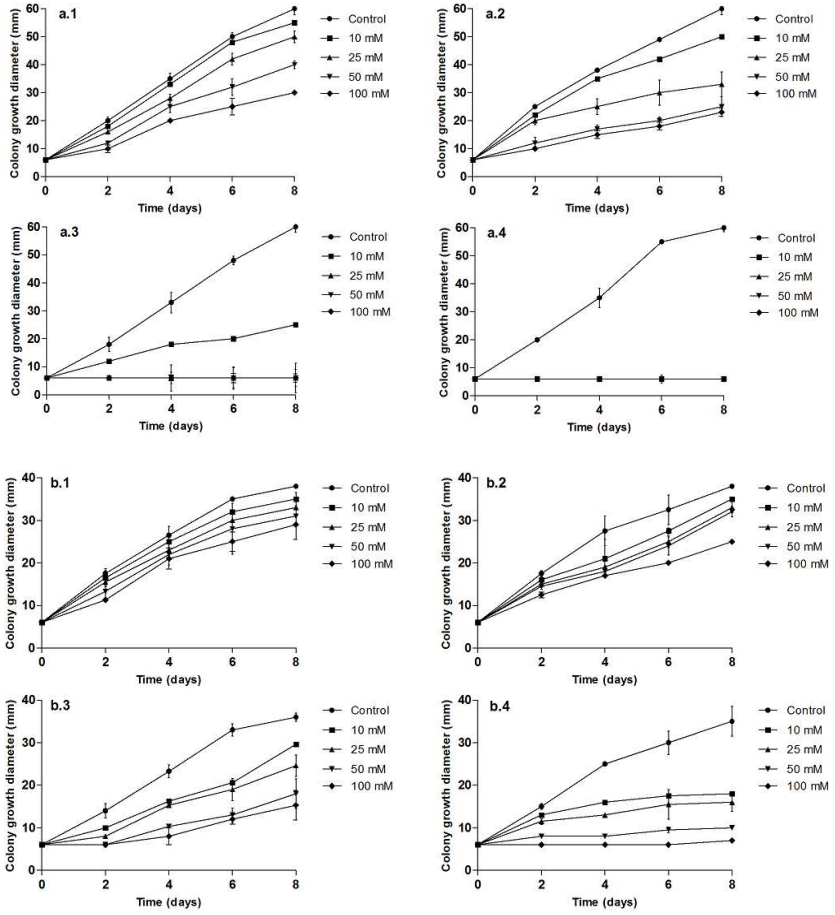
Figure S1. Characterisation of ZnO-NPs by (a) XRD plot and (b) TEM imaging showing NPs with mean diameter of 30 nm.

Antifungal and antimycotoxin activities of Zn-compounds

Antifungal

Our results showed that the effect of Zn-compounds on growth diameter of fungal colony was time and concentration dependent. Some treatments completely inhibited fungi growth. *F. graminearum* was significantly reduced by all treatments, but only completely inhibited by $ZnSO_4$ and $Zn(ClO_4)_2$ (Fig. S2a). Also A. flavus was significantly reduced by all treatments, but not completely inhibited after the 8th day of incubation (Fig. S2b). On the other hand, *P. citrinum* was most treatment sensitive, which was completed inhib-ited by ZnO-NPs, $ZnSO_4$ and $Zn(ClO_4)_2$ (Fig. S2c). Recent studies have showed antifungal activity of Zn-compounds, including ZnO-NPs. Sharma et al. (2011) found strong antifungal activity of ZnO-NPs synthetised by microwave method against plant fungus *Pythium debarynum* in the concentration of 10 mM. He et al. (2011) showed that 12 mM of ZnO-NPs was suffi-cient to completely inhibit the growth of *Botrytis cinerea* and *Penicillium expansum*. Still, ZnO showed inhibition of fungi growth only in high concentrations (100 mg mL^{1}) by conductimetric assay (Sawai & Yoshikawa, 2004). Antibacterial properties of $ZnSO_4$ also were studied, showing that their addition to glass-ionomer-based cements led to

significant inhibition of *S. mutans* growth (Osinaga et al., 2003). Zn chloride also showed inhibition on bacterial species (*Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *S. hyicus*, *E. faecalis* and *E. faecium*) (Aarestrup & Hasman, 2004). Moreover, Kumar et al. (2013) demonstrated the ZnO nanostructures antimicrobial activity against *S. aureus* and *E. coli*. In our study, the $ZnSO_4$ and Zn $(ClO_4)_2$ showed to be most effective treatments, inhib-iting completely the *F. graminearum* and *P. citrinum* growth in the concentration of 100 mM.



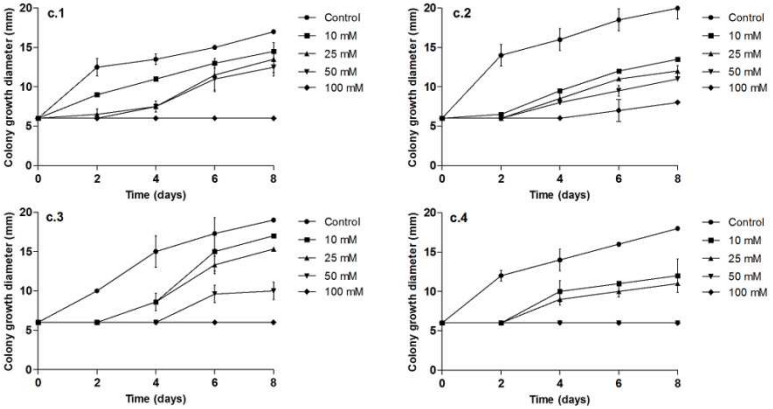


Figure S2. Antifungal activities of (1) ZnO-NPs, (2)ZnO, (3) ZnSO4 and (4) Zn(ClO4)2 against (a) *F.graminearum* (b) *A. flavus* and (c) *P. citrinum* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal). All treatments were statistically significant when compared with Control group ($P < 0.05$).

Antimycotoxin

The production of DON and AFB_1 by *F. graminearum* and *A. flavus* grown on Zn-compounds treatment was smaller when compared to controls. F. graminearum strain only produced DON when grown on ZnO medium treated. Others treatments inhibited fungal growth and their ability to produce DON. On the other hand, A. flavus was able to produce AFB_1 in almost all treat-ments. Despite this, the amount of AFB_1 produced by the treated fungi was significantly lower than Control. Regarding *P. citrinum*, it also produced CTR in almost all treatments, except for $Zn(ClO_4)_2$. The CTR production has no significant difference from control when treated with ZnO-NPs and ZnO; nevertheless, a signifi-cant decrease was observed with $ZnSO_4$ treatment (Fig. S3). The stress caused by the chemical treatment can lead to an increase in mycotoxin production by some fungi. Pacheco & Scussel (2007) also verified that *A. flavus* was able to produce high AFLs amounts sim-ilar to the Control in culture medium, when treated with selenium (Se) at different concentrations.

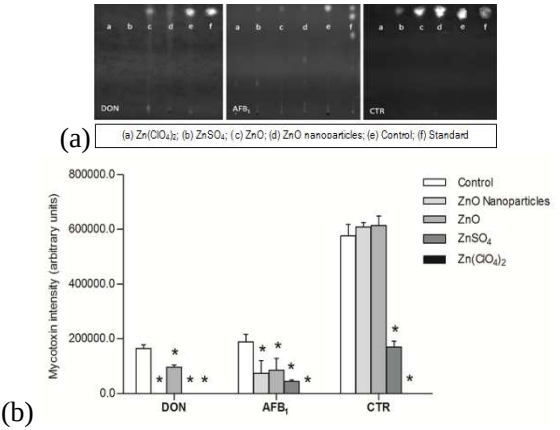


Figure S3. Antimycotoxin activities of Zn-compounds treated (100 mM) on toxigenic *F. graminearum* (DON), *A. flavus* (AFB_1), *P. citrinum* (CTR) and (a) showing TLC plates with mycotoxins by metodology; (b) data are shown as average values and standard deviation of mycotoxin intensity (arbitrary units - a.u.). Symbols indicate statistically significant when compared with Control group *P < 0.05.

Conidia production reduction

The conidia production by treated fungi was significantly reduced in almost all Zn-treatments (Fig. S4). There are several factors (external environment temper-ature, presence of water, chemical treatments) which can alter conidia production. The number of conidia can decrease in extreme conditions of growth, such as long-term storage at low temperatures (Aregger, 1992). For *F. graminearum*, the conidia production reduced in all Zn-compounds treatments. On the other hand, *A. flavus* and *P. citrinum* had their conidia production reduced only after some treatments, as ZnO-NPs, $ZnSO_4$ and $Zn(ClO_4)_2$. The treatments with $ZnSO_4$ and $Zn(ClO_4)_2$ were the best in terms of conidia production inhibition and fungi growth reduced. In general, conidia production can reflect the reproduction capacity and fungi development. Then, these results suggest that the effect of Zn-compounds on fungi growth could be related to their property alter reproduction capacity, in terms of conidia viability.

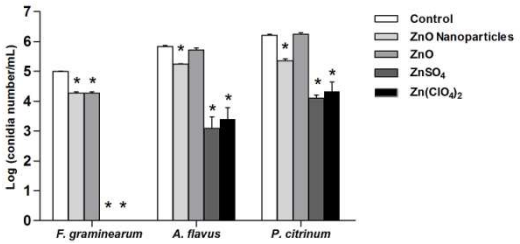
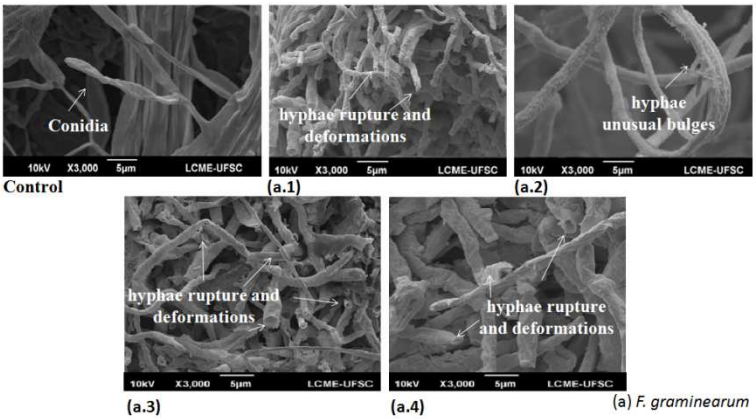


Figure S4. Conidia production of *F. graminearum, A. flavus* and *P. citrinum* on Zn-compounds media treated (100 mM). Data are shown as average values and standard deviation of log (conidia number per mL). Symbols indicate statistically significant when compared to Control group *P < 0.001.

Hyphae morphological alterations, mortality determination and ROS production

Morphological alterations

Due to reduction of their growth and ability to produce mycotoxins, we supposed that morphological changes on treated fungi could occur. To evaluate them, treated fungi hyphae at the highest Zn-compound concentration (100 mM) were submitted to SEM analysis. The fungi hyphae that received Zn-treatment showed dam-age such as: deformations, ruptures and unusual bulges that were not observed in the controls, that is, fungi that grew on medium without treatment. These data were observed with *F. graminearum* (Fig. S5a), *A. flavus* (Fig. S5b) and *P. citrinum* (Fig. S5c). SEM analyses confirmed the fungi cell membrane rupture resulting in possible reduction of the enzymatic activity of the micro-organism due to Zn-compounds treatments. Changes in the structure of the fungus were also observed using ZnO-NPs against *P. expansum* and *B. cinerea* (He et al., 2011). SEM analyses also can confirm changes and rupture of the fungal cell membrane in *F. verticillioides*, *A. flavus* and *P.citrinum* due to gold NPs presence in culture medium (Savi et al., 2012).



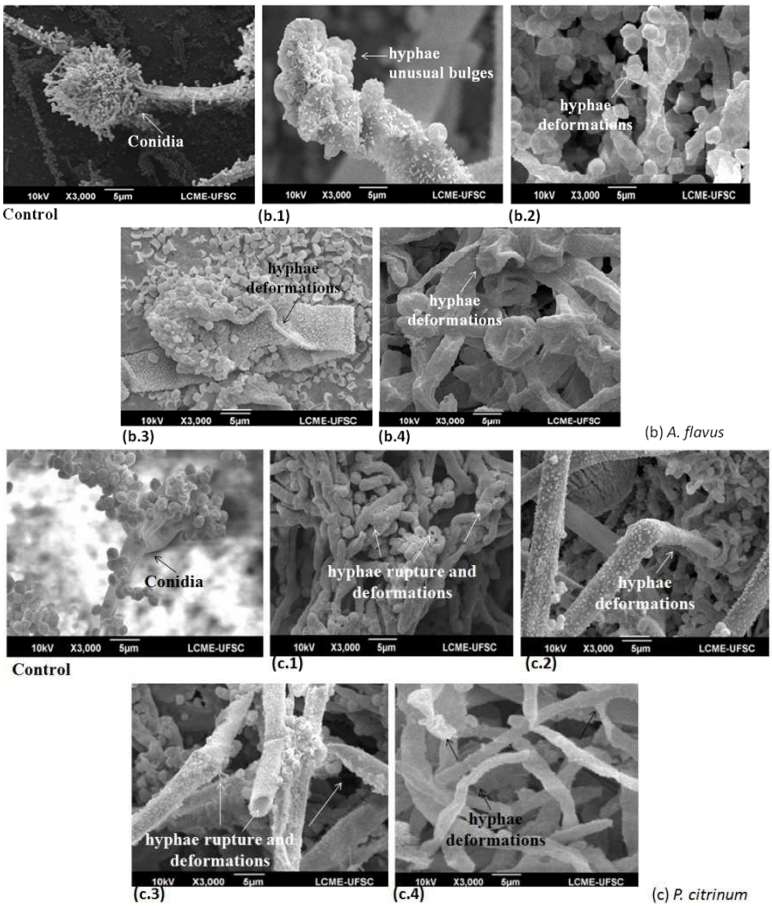


Figure S5. Effect of the Zn-compounds treatments on (a) *F. graminearum*, (b) *A. flavus* and (c) *P. citrinum* hyphae. [Control: no treatment; zinc- compounds treated (100 mM): (1) ZnO-NPs, (2) ZnO, (3) $ZnSO_4$, (4) $Zn(ClO_4)_2$, showing hyphae alterations]. SEM micrographs images in size of x3000.

Mortality determination

The Zn-compounds treatments altered the hyphae morphology, and these changes could be associated with cell damage that are not repaired conveniently (Semighini & Harris, 2010). A variety of factors, such as developmental signs, physical, chemical stresses and antifungal compounds, have been reported to trigger necrosis or apoptosis-like cell death in fungi (Sharon et al., 2009). Zn-compounds treatments can cause

biochemical changes in fungal cells, being capable of dis-rupting cells integrity. These changes can lead to cell death, being possible to detect by Evans Blue staining. Ours results show that normal hyphae (Control) presented an intact plasma membrane, being able to exclude Evans blue dye and remain their natural colour. However, Zn-treated hyphae were blue stained, suggesting the presence of dying hyphae that undergo plasma membrane lysis and became unable to exclude the dye. The fungi hyphae treated with $ZnSO_4$ and Zn $(ClO_4)_2$ were observed the strongest staining intensity with Evans blue (Fig. S6) indicating that these compounds inflicted higher damage to fungi hyphae comparative to others treatments.

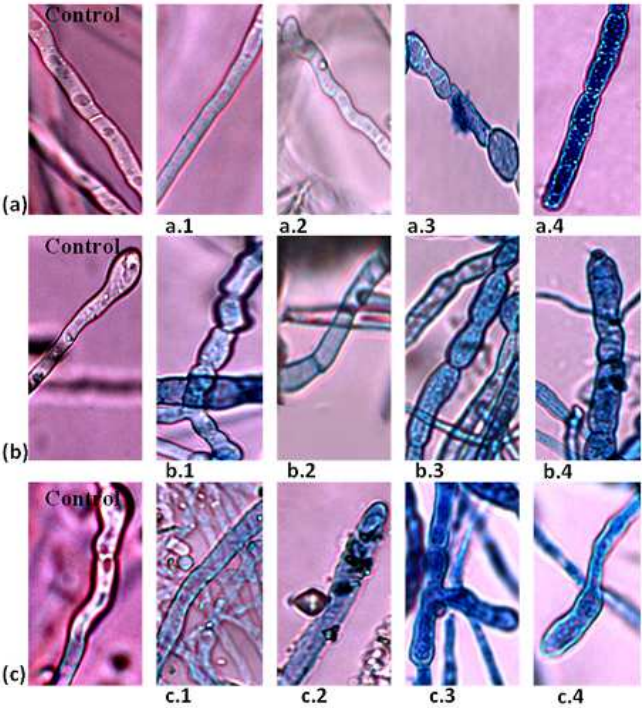


Figure S6. Effect of the Evans Blue dye distribution in the (a) *F.graminearum*, (b) *A. flavus* and (c) *P. citrinum* hyphae. [Control: no treatment – without coloration (hyphae alive); Zn-compounds treated (100 mM): (1) ZnO-NPs, (2) ZnO, (3) $ZnSO_4$, (4) Zn $(ClO_4)_2$, showing hyphae coloration (hyphae dead). OM images at x400.

Reactive oxygen species production

The hyphae mortality can be related to cell structure damages observed through the hyphae morphological changes. These alterations can increase dramatically the levels of ROS formation and be responsible for the fungi cell death (Avery, 2001; Valko et al., 2005). Some metals can also interact directly against the nucleic acids, as deoxyribonucleic acid (DNA), pro-ducing mutations and carcinogenesis and to induce the ROS formation. In the healthy aerobic cells, ROS has important roles in cell signalling and homoeostasis, being that their generation typically occurs at a controlled rate. However, under stress conditions, their production can be greatly increased. Apoptotic-like cell death has been reported in multicellular organ-isms, leading to exposure to ROS high-level formation (Semighini et al., 2006, 2008). To evaluate whether fungi cell death occurred due to the increase in intracellular ROS production, we utilised H_2DCFDA as a specific proof of general oxidative stress. H_2DCFDA is permeable to cell membrane and undergoes intracellu-lar conversion by nonspecific esterases to form nonfluorescence 2,7 dichlorofluorescein (DCFH). DCFH oxidises in the presence of ROS to form 2,7 dichlorofluorescein (DCF), which emits a highty green fluorescence (Cathcart et al., 1983). Our results pointed out to an increase in ROS production in the fungi hyphae treated with any Zn-compounds, being that fungi hyphae $ZnSO_4$ and $Zn(ClO_4)_2$ treated were observed a stronger fluorescence intensity (Fig. S7). ROS was also present in the fungi cell control, however, in quite small amounts and fluorescence intensity when com-pared to the treated ones. The action of Zn-compounds observed on the fungi hyphae morphology, mortality and ROS production can be related to the antifungal and antimycotoxins activities of the treatments utilised in this study against toxigenic fungi.

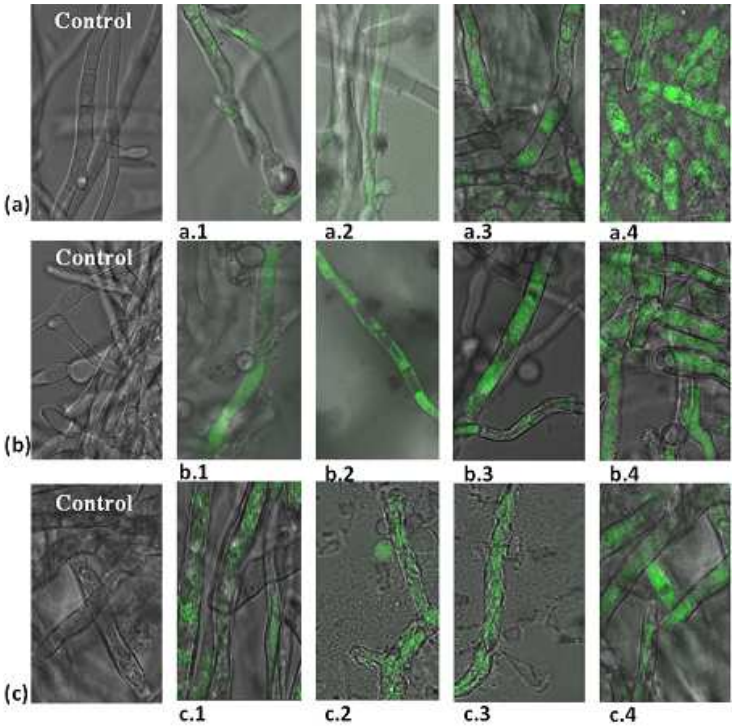


Figure S7. Effect of the H_2DCFDA on (a) *F. graminearum*, (b) *A. flavus* and (c) *P. citrinum* hyphae. [Control: no treatment (no ROS production); Zn-compounds treated (100 mM): (1) ZnO-NPs, (2) ZnO, (3) ZnSO4, (4) Zn(ClO4)2, showing hyphae fluorescence, development after ROS production]. COM images at x300.

Conclusion

Zn-compounds have advantages in relation to other antifungal compounds because are cheap, stable and nontoxic for the organism, if utilised in appropriate amounts. Moreover, they can present strong antifungal activity even at low concentrations. Zn-compounds presented significant results, being the bests with $ZnSO_4$ and $Zn(ClO_4)_2$ treatments, which were observed stronger antifungal and antimycotoxins activities. The fungal hyphae modifications were clearly observed. The Zn-compounds treatments interfered to the fungi cellular metabolism, on conidia (number reduction) and hyphae (morphological alterations, mortality and ROS production). These results suggest that Zn-

compounds could be further studied as an effective fungicide for agricultural and food safety applications, as for example, in food packaging to improve protection of foods against microbiological effects and developing active antibacterial and antifungal surfaces. Zn-compounds applications as antifungal must agree with tolerable upper limit for consumption, because that Zn residues may remain in food or migrate from food packaging into food. New studies are needed to investigate the feasibility of incorporating Zn-compounds into films and other packaging materials, as well as their application in agriculture, where mycotoxins are one of the main problems regarding contamination of foods.

Acknowledgments

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7 CAPÍTULO 5

Efeito dos Compostos de Zinco sobre *Fusarium verticillioides* com relação ao Crescimento das Colônias, Alterações nas Hifas, Conídios e Produção de Fumonisinas

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Effect of zinc compounds on *Fusarium verticillioides* growth, hyphae alterations, conidia, and fumonisin production

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Abstract

BACKGROUND: Several strategies are used to eliminate toxigenic fungi that produce fumonisinsin grains. *Fusarium verticillioides* can be controlled by the application of synthetic fungicides in the field or during storage. However, there may also be residuals, which may remain in the foods. Inorganic compounds such as zinc are cheap, stable and could present strong antifungal activity. Some Zn compounds can be utilized as dietary supplements and are authorized for the fortification of foods. Knowing the advantages and that low concentrations of Zn can have antimicrobial activity, our objective was to evaluate the effects of Zn compounds on the growth of *F. verticillioides* and the production of fumonisin and conidia. In addition, we aimed to verify that Zn compounds cause morphological alterations of the hyphae, mortality and production of reactive oxygen species.

RESULTS: Zn compounds efficiently reduced fungal growth and fumonisin production. Treatment using zinc perchlorate gave the best results. All treatments inhibited conidia production and caused morphological alterations of the hyphae. It was possible to observe cell death and production of reactive oxygen species.

CONCLUSION: Zn compounds have advantages compared to other antifungal compounds. In particular, they are non-toxic for the organism in appropriate amounts. They could be studied further as potential fungicides in agriculture.

Keywords: zinc compounds; *Fusarium verticillioides*; growth; fumonisins

INTRODUCTION

Fusarium verticillioides is a widely distributed pathogen and is reported as a species that infects all stages of plant development, having the ability to infect and cause tissue destruction in important crops (corn and wheat mainly). However, its scope is not limited to the crops on the field, as mycotoxins may be produced under storage conditions, although on a small scale, if under optimal conditions (e.g. high moisture content).[1]

The main toxins produced are the fumonisins (FBs), which are associated with several mycotoxicoses, including equine leukoencephalomalacia,[2,3] swine pulmonary edema[4,5] and experimental rats kidney and liver cancers.[6,7] Among the FBs, FB_1, FB_2 and FB_3 are the main grain contaminants detected. Generally, FB_1 makes up 70% of the total FBs produced, followed of FB_2 and FB_3.[8] The International Agency for Research on Cancer has classified FB_1 as a possible carcinogen to humans.[9]

The production of FBs in agricultural commodities depends upon several factors, such as the geographical region, season, and the environmental conditions under which the crops are grown. Tropical and subtropical regions are the most favorable for the development of field fungi on cereals and the production of these toxins.[10] Although cereals are important as substrates, moisture level and temperature are the critical abiotic factors regulating the growth of *F. verticillioides* and the production of FBs.[11] The presence of FBs has been reported in several foods worldwide, especially grains.[12–17]

Several strategies have been applied to eliminate toxigenic fungi FB producers in grains. *Fusarium* can be controlled by applying synthetic fungicides in the field. However, that treatment can be problematic due to the compounds toxicity on mammals and because their residues may remain in the foods.[18] Indeed, fungicide treatment post-harvest, including during grain storage can lead to serious health hazards for consumers.[19] Furthermore, the use of fungicides as chemical methods can develop resistance in the fungi.[20]

Therefore, the development of new antifungal agents that can assist in the current control strategies is essential. Inorganic compounds such as zinc (Zn) are stable, have been reported to be strong antimicrobial agents, apart from being less toxic, stable and cheaper than other products.[21] The most common inorganic zinc compounds are zinc sulfate ($ZnSO_4$), zinc perchlorate [$Zn(ClO_4)_2$] and zinc oxide (ZnO). In recent years ZnO in nanoscale particle size (NPs) also has received

special attention due to the special physical and chemical properties of the nanoparticles.[22–24] Some studies have reported the utilization of these compounds for antibacterial activity.[25–29] There are, however, only a few studies reporting their action against filamentous fungi[30–32] and no studies on the effects of these compounds either against *F. verticillioides* or on its toxigenicity.

It is important to emphasize that several zinc compounds have been utilized in dietary supplements for human consumption under different forms (zinc acetate/ chloride /citrate/ gluconate/ lactate/ oxide /carbonate / sulfate) which are considered as Generally Recognized as Safe (GRAS) and authorized also in human foods fortification by several international official boards[33,34] including approval by the European Commission (EC 2002). They are taken as supplements for their health benefits as zinc plays role in multiple enzyme systems related to the metabolism of protein, carbohydrates, fat and alcohol.[35]

Considering that (1) zinc compounds have advantages regarding effectiveness, safety and stability on anti-bacteria activity at low Zn concentrations; (2) lack of information of these compounds (including at NPs sizes) anti-fungal properties either for toxigenic species of *Fusarium* and FBs formation; and (3) the need for effective control and prevention methods against toxigenic *F. verticilioides* for application to field crops, this work reports an evaluation of the effects of three different zinc compounds (including ZnO at NPs size) on toxigenic *F. verticillioides* regarding: anti-fungi (colony growth inhibition) and possible anti-toxin (reduction of FBs) properties apart from conidia production and hyphae alterations (cell mortality and formation of reactive oxygen species) by using scanning electron microscopy (SEM), light microscopy (LM) and confocal optical microscopy (COM), respectively.

MATERIALS AND METHODS

Materials

Fusarium verticillioides, the toxigenic strain for FBs (FB_1 and FB_2), was obtained from the Food Mycology Laboratory of Mycotoxicology and Food Contaminants (LABMICO) culture collection at the Federal University of Santa Catarina, Florianopolis, SC, Brazil. $ZnSO_4$, $Zn(ClO_4)_2$ and ZnO were all of analytical grade, and purchased from Sigma – Aldrich (St Louis, MO, USA). ZnO at NP size (had a mean diameter of 30 nm). The reagents 2,7-

dichlorodihydrofluorescein diacetate (H_2DCFDA) and Evans blue dye were from Sigma–Aldrich; disodium hydrogen phosphate, monopotassium phosphate, Tween 80 and formaldehyde 37%, were from Synth (Diadema, SP, Brazil); sodium chloride and potassium, from Vetec (Duque de Caxias, RJ, Brazil); and *o*-phthaldialdeyde was from Merck (Barueri, SP, Brazil). The solvents methanol, ethanol and acetonitrile, all pro analysis (p.a.) were from Synth (Diadema, SP, Brazil); while methanol HPLC grade was from Montcada (Barcelona, Spain). FB standards (FB_1 and FB_2), 1 mg, crystals, and potato dextrose agar were from Sigma – Aldrich. Sabouraud liquid medium was from Himedia (Curitiba, Parana, Brazil). A Neubauer chamber was from Ciencor Scientific (Sao Paulo, SP, Brazil); the solid phase extraction (SPE) cartridge of quaternary amino N^+ C_{18} (capacity, 500 mg; vol, 6 mL) was from Applied Separations (Allentown, PA, USA) and stainless steel stubs

Equipment and apparatus

The following equipment for microscopy was used: a CH-Bl45-2 light microscope (Olympus, Shinjuku, Tokyo, Japan); a JSM-6390LV scanning electron microscope (Jeol, Peabody, MA, USA); a DMI6000B confocal microscope (Leica, Leider, IL, USA); and a JEM-2100 transmission electron microscope (Jeol).

Other equipment was: an X-ray diffractor (Cade-4; Enraf-Nonius, Eugene, OR, USA); critical point dryer (CPD 7501; Quorum Technologies, Guelph, Canada); gold coater (SCD500; Leica); high-performance liquid chromatograph with a fluorescence detector (321; Gilson, Middleton, WI, USA); autoclave (Phoenix; Araraquara, SP, Brazil); microwave oven (Philco, Sao Paulo, SP, Brazil); laminar flow cabinet (Veco, Campinas, SP, Brazil); fume cabinet (Quimis, Diadema, SP, Brazil); ultraviolet cabinet (Dist, Florianopolis, SC, Brazil); Eppendorf centrifuge (5415R; Eppendorf, Sao Paulo, SP, Brazil); and microbiological incubator (Quimis).

Methods

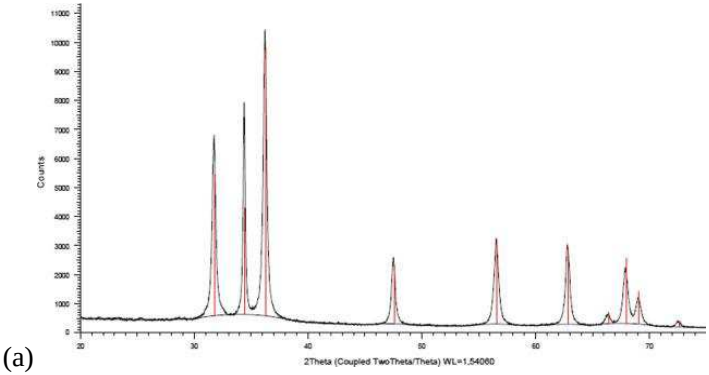
Preparation of zinc oxide at nanoparticle size

NPs were synthesized according to Sharma *et al.*[36] and characterized by the X-ray diffraction (XRD) patterns obtained utilizing a X-ray diffractor and field emission transmission electron microscopy

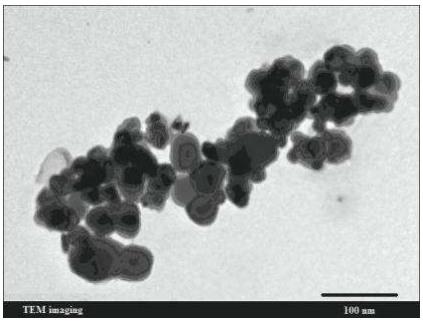
(TEM). The synthesized ZnO-NPs resulted in small particle size (mean diameter, 30 nm) and their XRD patterns are shown in Fig. 1a. All the diffraction peaks were in good agreement with those of the hexagonal wurtzite structure of ZnO (JCPDS card 36 – 1451). The sharp intensity peaks (between 30° and 40° theta scale) could be indexed to the wurtzite ZnO with high crystallinity.[37] Figure 1b shows TEM analysis with the ZnO-NPs nearly spherical geometry and mean diameter, calculated from XRD.[38]

Preparation of solutions of zinc compounds

Concentrations of 10, 25, 50 and 100 mmol L^{-1} were prepared for $ZnSO_4$ (Group I), $Zn(ClO_4)_2$ (Group II), ZnO (Group III) and ZnO-NPs (Group IV) by diluting each compound in water.



(a)



(b)

Figure 1. Nanoparticles of ZnO synthesis and characteristics: (a) X-ray diffraction plot with the ZnO maxima; and (b) imaging of nanoparticles

(NPs) by transmission electron microscopy. Mean size of NPs, 30 nm (scale: 100 nm).

Effect of zinc compounds on fungal growth

The *F. verticillioides* treatment for the evaluation of Zn anti-fungal activity was performed according to the method of Fraternale *et al.*[39] *F. verticillioides* Zn treatments were divided into the following groups: Group I, II, III and IV [for $ZnSO_4$, $Zn(ClO_4)_2$, ZnO and ZnO-NPs, respectively] keeping a Group C as control (no Zn treatment) and prepared as follows: different solutions were added to autoclaved potato dextrose agar (PDA) media into Petri dishes, keeping one as Control (PDA no Zn-treated). A disc (6 mm) of *F. verticillioides* mycelial material, taken from the edge of 7-day-old fungal cultures was placed at the center of each Petri dish containing the culture media Zn treated and incubated at 25 ºC for 8 days. The efficiency of treatment with the Zn compounds was evaluated until the 8th day of incubation by measuring the diameter (mm) of the fungal colony.

Effect of zinc compounds on fumonisin production

The *F. verticillioides* strain was grown on PDA media with the zinc compounds at the highest concentration together with the Control Group at the same temperature as above but keeping it at a higher moisture content (80%). The grown (20 days) fungi colony plus its PDA media were transferred to a separation funnel for extraction of FBs by using methanol–water, followed by filtration and cleaning up the extract by using solidphase extraction (SPE).[40] The FBs were eluted and the extract evaporated with nitrogen at 60 ºC, followed by derivatization with *o*-phthaldialdeyde and quantification by LC-FLD at 335 and 440nm for excitation and emission, respectively. The mobile phase was methanol–sodium dihydrogen phosphate solution (77:23). The limit of detection (LOD) and limit of quantification (LOQ) for the method were 0.04 and 0.05, and 0.21 and 0.24 $\mu g\ kg^{-1}$ for FB_1, FB_2, respectively.

Effect of zinc compounds on conidia production

The *F. verticillioides* strain was grown on PDA media containing the zinc compounds and Control for 8 days at 25 ºC. The colonies fungi mycelia were transferred to a tube containing a solution of 0.89% NaCl and 0.1% Tween 80 then and shaken to detach the fungi

conidia. A drop of this conidia suspension was counted in a Neubauer chamber by LM at × 400 magnification.[41]

Morphology

The treated *F. verticilloides* mycelia PDA sections were collected, the fungi were fixed with formaldehyde, washed with phosphate-buffered saline (PBS) and dehydrated with alcohol solution (30, 60, 80, 90 and 100%, keeping the mycelia for longer at 100%). Then it was taken for critical point drying according to Bray.[42] Then, fungal mycelia were prepared for SEM analysis as follows: strains were fixed onto stubs, placed on the gold coater holder, a vacuum (up to 10^4 mbar) was applied, and the sample coated with a 1.40 nm layer of gold.

To observe the *F. verticillioides* mycelia by SEM, stubs with Au-coated fungi strains were transferred to the scanning electron microscope, submitted again to a vacuum and the cells were visualized, identified at different magnifications and registered by micrographs (taken at a voltage of 0.5 – 30 kV).

Mortality

The *F. verticillioides* spores were incubated in Sabouraud liquid medium for 24 h at 28 ºC for hyphae formation, and later 0.05% transferred to this same medium containing the Zn compounds (highest concentration, 100 mmol L^{-1}) for another period of incubation of 24 h at 28 ºC. Hyphae were centrifuged, soaked in 0.05% Evans blue dye solution and left stand for 5 min. Finally, hyphae were washed three times with PBS to remove excess dye. The resultant sediment was verified with LM at ×400 magnification. Hyphae were observed according to their staining differences.[43]

Production of reactive oxygen species

To evaluate the levels of reactive oxygen species (ROS) in zinc-treated *F. verticillioides* hyphae, the H_2DCFDA probe, which reacts with ROS and becomes fluorescent, was utilized. The fungal spores were incubated in Sabouraud liquid medium for 24 h at 28 ºC, for hyphae formation. Then, the hyphae were incubated in the same medium containing zinc compounds (at the highest concentration: 100 mmol L^{-1}) for 24 h at 28 ºC. The fungi suspension was centrifuged and treated with 40 µmol L^{-1} H_2DCFDA for 30 min at 28 ºC in the dark. Finally, the

sediment was washed three times with PBS and analyzed by COM at × 300 magnification. The production of ROS was observed by the presence of green fluorescent hyphae.[44]

Statistical analysis

Data on colonies fungi growth, conidia production and anti-mycotoxin activity were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-test. All analyses were expressed as mean ± SD and P values < 0.05 were considered statistically significant.

RESULTS

From the data obtained on the Zn anti-fungal/anti-FBs properties, *F. verticillioides* conidia production and hyphae alterations, all treatments (depending on the concentration applied and type of Zn compounds utilized - Groups I to IV), inhibited fungus growth and FBs formation to same extent. They also caused hyphae morphological alterations leading to mortality and ROS production, in different proportions though, some Zn being compounds more effective than others. Figure 2 and Table 1 show data regarding the effects of treatment with zinc compounds on growth of *Fusarium*/FBs formation. Figure 3 and Fig. 4 show alterations in fungi structure as observed by SEM, LM and COM.

Anti-fungal properties

Treatment with all the zinc compounds at all concentrations applied were able to significantly inhibit *F. verticillioides* growth from ≥ 25 mmol L^{-1} concentration, with $Zn(ClO_4)_2$ (Group II) being the treatment that completely inhibited fungi colony growth at 100 mmol L^{-1}. The other zinc treatments still showed some *Fusarium* growth throughout all concentrations, with the highest being 19 > 18.3 > 14.5 mm (ZnO > $ZnSO_4$ > ZnO-NPs) colony diameters for Groups III, I and IV, respectively, in contrast to the Group Control of 45 – 49.5 mm (Fig. 2).

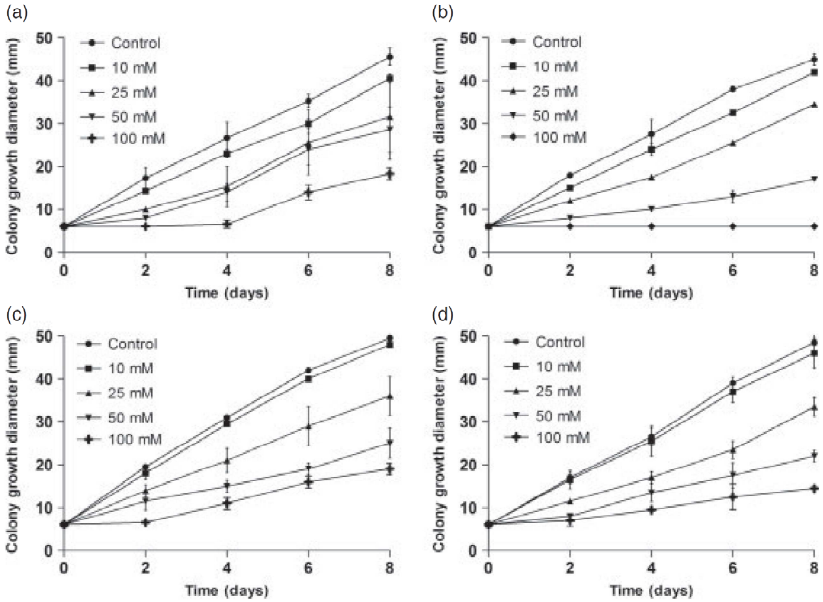


Figure 2. Anti-fungal activity of (a) $ZnSO_4$, (b) $Zn(ClO_4)_2$, (c) ZnO and (d) ZnO-NPs, against *F. verticillioides* on potato dextrose agar (PDA) at different concentrations (data are shown as average values and standard deviation of fungal diameter).

Anti-mycotoxin properties

All treatments with zinc compounds completely inhibited FB_1 production by *F. verticillioides* when compared to Control Group (FB_1, 464 μg kg^{-1}). On the other hand, FB_2 production was inhibited completely only by $ZnSO_4$, $Zn(ClO_4)_2$ and ZnO-NPs treatments. ZnO treatment was quantitatively less effective, although better (FB_2, 62 μg kg^{-1}) than the Control Group (FB_2, 90 μg kg^{-1}). Table 1 shows the inhibition of FBs production (data are shown as average values and standard deviation).

Table 1. Anti-mycotoxin activity of zinc compounds on *Fusarium verticillioides*, as determined by the production of fumonisins FB_1 and FB_2

	Zinc treatments			Fumonisins (μg kg^{-1}) (reduction, %)		
Group	Compound	Particle size	Concentration (mmol L^{-1})	FB_1	FB_2	FB_{total}
C	Control	NA	0 (no Zn treated)	464 ± 5.65 (NA)	90 ± 1.41 (NA)	554 ± 7.06 (NA)
I	$ZnSO_4$	Reg	100	< LOD (100)	<LOD (100)	<LOD (100)
II	$Zn(ClO_4)_2$	Reg	100	< LOD (100)	<LOD (100)	<LOD (100)
III	ZnO	Reg	100	< LOD (100)	62 ± 3.53 (68.9)	62 (68.9)
IV	ZnO-NPs	Nano (30 nm)	100	< LOD (100)	<LOD (100)	<LOD (100)

FB_1 and FB_2 production was analysed by liquid chromatography with fluorescence detection (335 and 440 nm for excitation and emission, respectively).
NA, not applicable.
LOD, limit of detection (0.04–0.05 μg kg^{-1}). Note that the limit of quantification was 0.21 and 0.24 μg kg^{-1} for FB_1 and FB_2, respectively.
NPs, nano-particle size.
Reg, regular size (> 100 nm).

Effect on conidia production

The four groups of treatments with zinc compounds, including ZnO of regular size and at NP size (Groups III and IV), significantly reduced *F. verticillioides* conidia production from a total of 5.84 log(conidia number) mL^{-1} (Control Group) to no conidia production by the Group II [$Zn(ClO_4)_2$] treated. Thus the efficiency of conidia production upon treatment of *Fusarium* with zinc compounds decreased as follows: $Zn(ClO_4)_2$ > $ZnSO_4$ > ZnO-NPs > ZnO. Figure 3 shows details regarding the effects of zinc treatment on the production of *F. verticillioides* conidia.

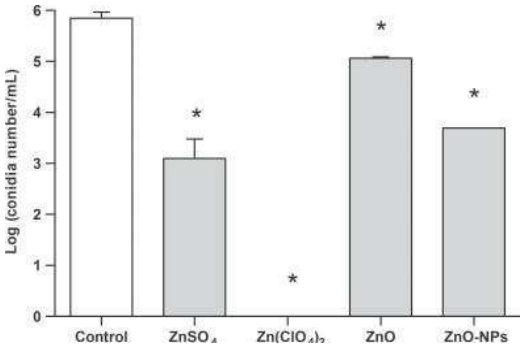


Figure 3. Conidia production of *F. verticillioides* after treatment with zinc compounds at 100 mmol L^{-1}. *Statistically significant when compared to Control, $P<0.05$; data are shown as the average value; standard deviation of log (conidia number) mL^{-1}.

Hyphae alterations: morphology, mortality and production of reactive oxygen species

Morphology

The reduction in growth of *F. verticillioides* observed upon treatment with zinc compounds can be caused by morphological and metabolic changes that occur during the formation of fungi structure (conidia and hyphae). To verify possible morphological alterations, SEM was used to observe fungi treated at the highest concentration (100 mmol L^{-1}) of zinc compounds. *F. verticillioides* treated with zinc compounds showed hyphae alterations, such as deformations and ruptures, which were not observed in the Control Group (fungi that grew without any zinc in the medium). However, alterations in conidia structure after zinc treatment were not found (Fig. 4a).

Mortality

The chemical stress caused by zinc treatment may lead to rupture of fungal cell integrity and then to cell death, which could be detected by the Evans blue staining test. Control Group hyphae presented an intact plasma membrane, able to exclude the Evans blue dye and remain the natural color of hyphae. On the other hand, hyphae treated with zinc compounds showed blue staining, suggesting the presence of dying hyphae in which cell integrity had been disrupted. Hence the hyphae were unable to exclude the dye and consequently were stained deep blue (Fig. 4b).

Production of reactive oxygen species

To evaluate whether fungal cell death occurred due an increase in the formation of intracellular ROS, H_2DCFDA was applied as specific proof of general oxidative stress. H_2DCFDA is transported across the cell membrane to the inner cell and deacetylated by esterases to form the non-fluorescent 2,7-dichlorofluorescein (DCFH), which remains trapped inside the cells. If ROS are present, DCFH oxidizes to form 2,7-dichlorofluorescein (DCF), which emits a strong greenish fluorescence.[43] Our results showed that ROS were present in the Zn-treated fungi hyphae and its production increased, as shown by the strong intensity green fluorescence (Fig. 4c). The $Zn(ClO_4)_2$ treatment had a high fluorescence increased for ROS production. Despite this, all zinc treatments showed increasing formation of ROS when compared to the control group.

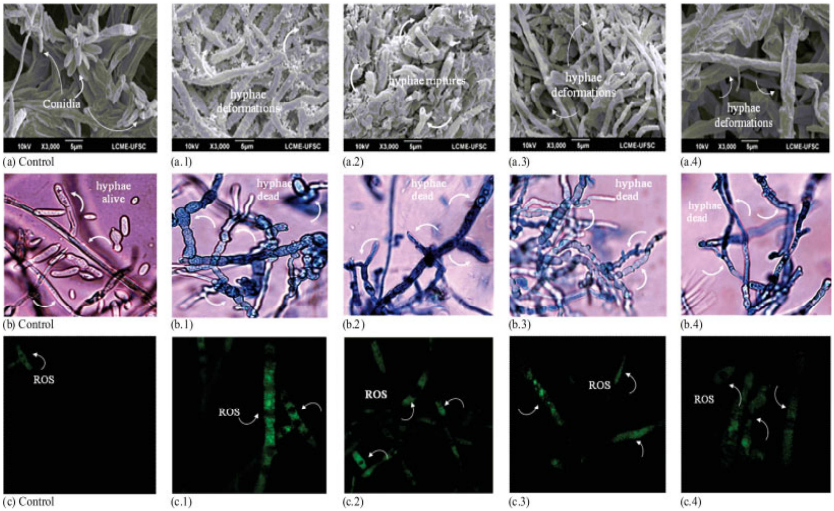


Figure 4. Effect of zinc treatments on *F. verticillioides*, showing hyphae: (a) alterations by scanning electron microscopy; (b) mortality after Evans blue staining, by light microscopy; (c) fluorescence with reactive oxygen species (ROS) production after H_2DCFDA reaction, by confocal optical microscopy. Treatment groups: Control (no Zn treated); 1, $ZnSO_4$; 2, $Zn(ClO_4)_2$; 3, ZnO; 4, ZnO-NPs. Concentration 100 mmol L^{-1}.

DISCUSSION

FBs are a series of mycotoxins mainly produced by *Fusarium* species, especially *F. verticillioides*, which cause contamination of plants and grains in the field. The consumption of food and products contaminated with *Fusarium* has been correlated with the development of an increased risk of diseases which can have an economic impact on agriculture, as well as an effect on human and animal well-being. Therefore, it is important to develop new anti-fungal agents that may replace current control strategies. Zinc compounds present strong anti-microbial activity at low concentrations. In addition, they have been be utilized as food supplements, especially as ZnO and $ZnSO_4$ which are also authorized for food fortification.[33,34]

Recent studies have shown the anti-fungal activity of zinc compounds, including ZnO-NPs. Sharma *et al.*[36] found strong anti-

fungal activity of ZnO-NPs synthesized by a microwave method against plant fungus (*Pythium debarynum*) at a concentration of 10 mmol L^{-1}. There are a few studies that also demonstrate anti-fungal activity against toxigenic filamentous fungi in food. He *et al.*[32] showed that 12 mmol L^{-1} of ZnO-NPs was sufficient to completely inhibit the growth of *B. cinerea* and *P. expansum*; however, the authors did not analyze the toxin concentration or its possible reduction. On the other hand, in another study, ZnO showed inhibition of fungi growth (*A. niger* and *Rhizopus stolonifer*) only at concentrations greater than 100 mg mL^{-1}, by conductimetric assay though.[30] There are no studies demonstrating the anti-fungal activity of zinc compounds against filamentous FBs producing fungi, or with the *F. verticillioides* strain used in this work.

Other studies reported treatments with antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, trihydroxybutyrophe-none and propylparaben) which were promising for *F. verticillioides* growth control group and FBs production.[45] On the other hand, the addition of a fungicide (quintozene, fludioxonil, metalaxyl-M) to the culture medium increased FB_1 mean levels compared to the control group of *F. verticillioides*.[46] In our results, $ZnSO_4$, $Zn(ClO_4)_2$ and Zn-NPs showed the best anti-mycotoxin activity, inhibiting both FBs produced by that *Fusarium* species (< method LOD).

When fungus is exposed to extreme adverse conditions of growth (i.e. chemical treatments, low temperature and the presence of high amounts of water) including long-term storage,[47] the number of conidia formed can be reduced. The treatments with $Zn(ClO_4)_2$ and $ZnSO_4$ were the best inhibitors of conidia production when applied at the highest concentration (100 mmol L^{-1}). Conidia production reflects the capacity for fungi development, and this is mirrored by our data, especially for the $Zn(ClO_4)_2$ (Group II) which showed the highest efficiency against both fungi growth and conidia production.

In addition to reducing the reproductive capacity, studies also showed that chemical treatments can induce cell death of yeast[48–51] and filamentous fungi.[52,53] Therefore all treatments with the zinc compounds caused damage to *F. verticillioides* in the current work, leading to hyphae cell death (intense blue color) and consequently a reduction in fungi growth. The fungi cell death can occur due to metabolism changes and general oxidative stress, as for example, the increase in the formation of intracellular ROS. The formation of ROS plays an important role in healthy aerobic cell signaling and homeostasis. Its production typically occurs at a controlled rate; however, under chemical stress, it can be greatly increased. That behavior is responsible

for fungi cell death due to apoptotic-like cell and nucleic acids mutations/carcinogenesis production. The amounts of ROS can be estimated by fluorescence detection and also quantified by fluorescence-activated cell sorting in single cells or by relative fluorescence measurements in the mycelia.[49,54–56] In this work, all treatments with zinc compounds showed the formation of increasing amounts of ROS when compared to the Control Group.

This is the first study to utilize zinc compounds as anti-fungal agents against *F. verticillioides*, and as anti-fumonisins, showing combined effects on conidia reduction, hyphae alterations, mortality and ROS formation. Data suggest that zinc compounds could be further utilized as effective fungicides in agricultural crops and for food safety applications against field fungi (*F. verticillioides*) – especially $ZnSO_4$, which presented the highest optimal anti-fungi activity – and is already authorized for foods fortification application: it is thus safe and non-toxic for plants, humans and animals, in adequate amounts. Furthermore, zinc compounds have advantages compared to other anti-fungal compounds because they are stable for the organism and are less expensive. Thus pilot studies will be performed to determine their possible application in agriculture (plant/grains in the field) and post-harvest, as anti-fungal and antimycotoxin agents.

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8 CAPÍTULO 6
Eficiência dos compostos de zinco no controle de Fusarium head blight (*Fusarium graminearum*) e formação de deoxinivalenol em trigo (*Triticum aestivum* L.)

ARTIGO SUBMETIDO: Savi, Geovana Dagostim; Piacentini, K.C.; Ramos, S.R.; Costa, M.E.B.; Santos, C.M.R.; Scussel, V.M. Efficacy of Zinc Compounds in controlling Fusarium head blight and deoxynivalenol formation in wheat (*Triticum aestivum* L.)

Efficacy of Zinc Compounds in controlling Fusarium head blight and deoxynivalenol formation in wheat (*Triticum aestivum* L.)

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Abstract

New strategies of control using Zinc Compounds in addition to conventional treatment could increase the efficiency against Fusarium head blight (FHB) in wheat. The efficiency of Zinc Compounds (zinc sulfate - $ZnSO_4$ and zinc oxide - ZnO in regular and nano size, respectively) on wheat plant were evaluated against the *F. graminearum* growth and DON formation. The plants were *F. graminearum* inoculated (10^6conidia/mL) and Zn-compounds (100mM) treated at the anthesis stage (in their spikelets). When wheat plants reached maturation, grains were harvested and evaluated for *Fusarium* colony susceptibility, DON formation, scanning electron microscopy (SEM) observation, followed by possible remaining Zn residues determination. The treated Groups with $ZnSO_4$ and ZnO-NPs showed a reduction of *F. graminearum* colonies growth ($2.6x10^3$ and $5.8x10^2$ CFU g^{-1}), when compare to Control ($2.8x10^3$ CFU g^{-1}). The same occurred for DON formation, i.e. the toxin was reduced from 1063.22 µg kg^{-1} (Control: no Zn-treated) to 721.16 µg kg^{-1} and no detected (method LOD: 66.7 µg kg^{-1}) for both treated Groups, respectively. As expect, the SEM images of wheat microstructures showed the characteristics *F. graminearum* hyphae and conidia on the grains pericarp surface, however, the treatment did not developed any damage to wheat grains. Besides, Zn remained within international recommended Zn level for consumption.

Keywords: Fusarium head blight, DON, wheat, zinc, control

1 Introduction

Fusarium graminearum is the primary causal agent of Fusarium head blight (FHB) disease on wheat spikes. That disease occurs primarily through the inoculum of *F. graminearum* (syn. *Giberella zeae*) from ascopores (located in the perithecia produced on wheat crop residues). The conidia are carried by wind over long distances and rest on the anthers during the flowering period, infecting the plant and causing depigmentation of affected spikelets (from green to white/brown/yellowish color). However, if the environment is highly moist, the pathogen produces massive number of conidia which appear as pink color and are a high source of inoculums (*Lima*, 2004).

Among the mycotoxins associated with FHB from the trichothecenes group, deoxynivalenol (DON) is the most commonly found in the wheat grains worldwide (*Ennouari* et al., 2013; *Santos* et al., 2013). DON acts as a virulence factor and is often found in the dispersing *F. graminearum* after initial infection of the wheat plant. The invasion of the kernel by *Fusarium* destroys the cell walls and starch granules. It also affects the endosperm storage proteins, resulting in a poor quality product (*Scussel* et al., 2011). Moreover, human and animal exposure to DON through the ingestion of contaminated food can induce to toxic effects such as immunosuppression, neurotoxicity and teratogenicity. Indeed, high doses causes more acute effects such as vomiting, rectal bleeding and diarrhea, while at chronic ingestion of DON in low doses causes anorexia and reduced growth (*Pestka*, 2007).

Since 2011 the Brazilian government regulation set the maximum tolerable level (MTLs) for DON in whole wheat grains at 2000 µg kg^{-1}. That limit will decrease over time to allow grain producers and the industry to adjust to that regulation without causing a shortage of wheat. As from January 2014, DON MTL for whole wheat grain was set at 1500 µg kg^{-1}, and from January 2016, it will decrease to 1000 µg kg^{-1} (*Brazil*, 2011). Despite that, a new regulation extended the time for that MTL to be achieved in 2017 (*Brazil*, 2013). Nowadays, the lower limit of DON is at 1250 µg kg^{-1} established by the European Commission for unprocessed cereals and 1750 µg kg^{-1} for unprocessed durum wheat (*EC*, 2006).

Synthetic fungicides can be used to control *F. graminearum* contamination on the field. However, that treatment has not been effective enough and has disadvantages due to, some of them, be highly toxic to mammals. Furthermore, the use of fungicides as chemical methods can lead to development of fungi resistance. Therefore, the

discovery of new antifungal agents that can assist in the current control strategies are essential (*Savi* et al., 2012; *Savi* et al., 2013a,b). The research interest on inorganic compounds such as zinc (Zn) derivatives is increasing (*Savi* et al., 2013a,b), as they are non-toxic in appropriates amounts and present strong antimicrobial activity at low concentrations. Indeed, Zn is an essential element for the human body and has been utilized in dietary supplements. Some salt forms, such as Zn acetate, chloride, citrate, carbonate, lactate and sulfate, as well as gluconate and oxide, are considered as *Generally Recognized as Safe* (GRAS) and authorized for the fortification of foods (*FDA*, 2011; *ODS*, 2011).

Besides, in the recent years, the Zn-compounds in nanoparticle sizes (NPs), also have received special attention due to their interesting physical chemical properties and biological application potential as antimicrobial agents. Nanotechnology has benefited the area of food safety, mostly through the development of highly sensitive biosensors for pathogen detection and development of novel antimicrobial solutions. Despite the NPs advantages, a clear understanding of the possible health effects are still unavailable, resulting in limitation to its widespread use, especially in the area of food security (*FDA*, 2012).

The suggested mechanism for the Zn-compounds antibacterial activity can be based on the reactive oxygen species (ROS) formation, which disrupt the integrity of the microbial cell membrane, assisting on the microbial enzyme bodies damage, thus killing the pathogenic microbe. Regarding fungi, only a few studies have been conducted (*He* et al., 2011; *Savi* et al., 2013a,b). In the study performed *in vitro* by *Savi* et al. 2013a, Zn-compounds ($ZnSO_4$ - zinc sulfate and ZnO-NPs - zinc oxide nanoparticles) showed antifungal and antimycotoxin activities against *F. verticillioides* and fumonisins, moreover, was observed hyphae morphology alterations, mortality and ROS production on treated fungi caused by treatment. The same authors (2013b), studied their effect on *F. graminearum* and DON production, reporting significant results *in vitro* to fungi and toxin control.

Considering that, the antifungal properties of Zn-compounds need to be further investigated in order to assist in the current FHB plant control strategies (especially on wheat spikes at anthesis stage) and so to avoid DON formation in the grains: this work evaluated the efficiency of $ZnSO_4$ and ZnO treatments (at regular and NP sizes, respectively) during the wheat plant growth, regarding *F. graminearum* growth and DON formation. In addition, any possible effect on the grains microstructures were observed by scanning electron microscopy (SEM), as well as the Zn remaining residue on wheat plant.

2 Materials and Methods

2.1 Materials

2.1.1 Samples

Wheat seeds from the 2012 crop, obtained from the Brazilian Agricultural Research Corporation (Embrapa Wheat, Passo Fundo, RS, Brazil), cultivar TBIO Pioneiro, without contamination by *F. graminearum* and DON (method LOD/LOQ: 66.7/119.1 $\mu g\ kg^{-1}$).

2.1.2 Fungi strain

Toxigenic *F. graminearum*, DON producer, obtained from the culture collection of the Food Mycology Laboratory of Mycotoxicology and Food Contaminants (LABMICO) from Federal University of Santa Catarina, Florianopolis, SC, Brazil.

2.1.3 Culture media, chemicals and other materials

Culture media: potato dextrose agar (PDA) and peptone bacteriology media, purchased from Himedia (Curitiba, Parana, Brazil); *Chemicals:* DON standard, $ZnSO_4$ and ZnO, obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA); acid nitric, acetonitrile (LC grade), methanol, sodium chloride (NaCl), tween 80 and chloramphenicol, obtained from Vetec (Duque de Caxias, RJ, Brazil). High-purity Milli-Q water (18.2 MΩ/cm) from a Millipore Synergy system (MA, USA) and *Other materials:* immunoaffinity columns (IAC) DON-Test from Vicam (Milford, MA, USA).

2.1.4 Instruments

X-ray diffraction (XRD) system, model Cade-4, Enraf (Nonius-Eugene, OR, USA); field emission transmission electron microscopy (TEM), model JEM-2100, Jeol (Peabody, MA, USA); light microscopes (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); scanning electron microscopy (SEM), Jeol (Peabody, MA, USA); atomic absorption spectrophotometer, Shimadzu (Nakagyo, Kyoto, Japan); muffle furnace, Quimis (Diadema, SP, Brazil); autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); rotary shaker, Marconi (Piracicaba, SP, Brazil); microbiological incubator, Quimis (Diadema, SP, Brazil); drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil); water activity (a_w) meter, Aqua-

Lab 4TE, Decagon Devices (Sao Jose dos Campos, SP, Brazil); Manifold, Phenomenex (Madrid Avenue, Torrance, USA). HPLC system, model 321, Gilson (Middleton, WI, USA) equipped with a isocratic pump model 805, manual injector (20 µL loop) and with ultraviolet-visible (UV) detector, model 118 (set a 218 nm); C_{18} reversed-phase chromatographic column (250 x 4.60 mm with 4µm particle size), Synergi, Fusion-RP 80, Phenomenex (Madrid Avenue, Torrance, USA).

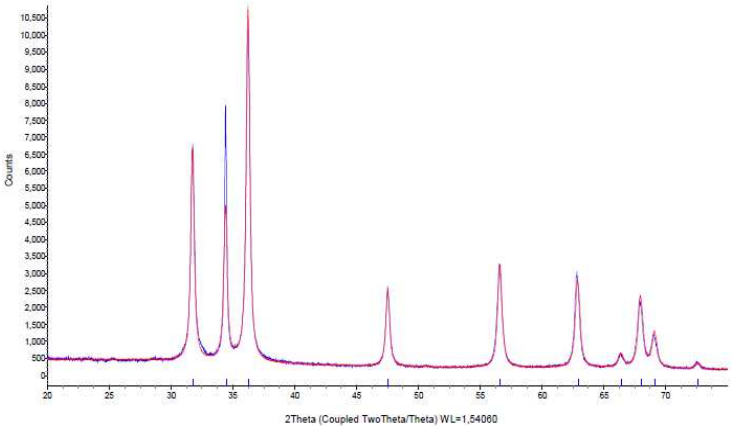
2.2 Methods

2.2.1 Fungal Spore Preparation

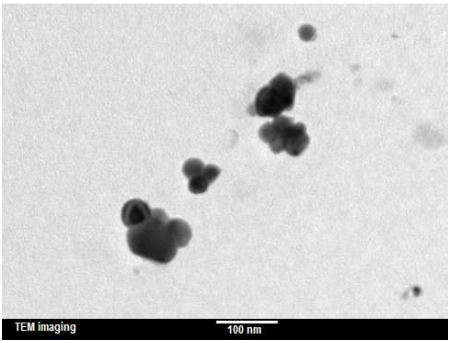
The toxigenic *F. graminearum* strain was grown on PDA medium at 25 ºC for 7 days (Nelson et al. 1983). The growth colonies of this fungi were taken from the culture edge (6 mm) and transferred to a 0.8 % NaCl plus 0.1 % Tween 80 solution tube, then stirred for fungi conidia detachment. The suspension was diluted in 0.8 % NaCl to obtain a 10^6 conidia mL^{-1} concentration. The conidia counting was performed with a Neubauer chamber by LM at x400 magnification (*Marques* et al., 2004).

2.2.2 Zn-compounds Preparation

ZnO-NPs were synthesized according to *Sharma* et al. (2011) and characterized by the XRD patterns obtained utilizing a X- Ray diffractor and TEM (Fig. 1A and B). The synthesized ZnO-NPs resulted in small particle size (mean diameter: 30 nm). The sharp intensity peaks (between 30º and 40º theta scale) could be indexed to the wurtzite ZnO with high crystallinity (*Guo* et al., 2005). TEM analysis showed ZnO-NPs nearly spherical geometry and mean diameter, calculated from XRD. The concentrations of 100 mM were prepared for ZnO-NPs and $ZnSO_4$, by diluting each compound in water.



(A)



(B)

Figure 1: Characterization of ZnO-NPs: (A) X-ray diffraction plot and (B) transmission electron microscopy NPs imaging, showing mean diameter of 30 nm size.

2.2.3 Wheat Crop

The field trials experiments were carried out at the Federal University of Santa Catarina, Brazil, in the Center for Agricultural Sciences (27°35'S latitude, 48°34'W longitude, 1.84 m altitude) during the wheat growing season in the months of August to November, 2013. The seeding was performed manually into individual plots with 5.5 m long and 1.7 m wide, consisting of nine rows with spacing of 0.2 m between them and 70 seeds per linear meter. The experimental area had a soil with 0-20 cm layer of organic matter: 2.1 %; clay: 16 %; water pH: 5.7; SMP index: 6.3; calcium (Ca): 5.0 cmolc dm^{-3}; magnesium (Mg): 0.7 cmolc/dm^{3}; potassium (K): 92 mg dm^{-3}; phosphorus (P): 145.4 mg dm^{-3}. The fertilization was performed according to the Commission of Chemistry

and Soil Fertility of the South Regional Center of the Brazilian Society of Soil Science (2004), with the application of 90 kg N/ha (nitrogen per hectare); 22.5 kg P_2O_5/ha (phosphorus pentoxide) and 35 kg K_2O/ha (potassium oxide) in the soil. In the field, during period of wheat crop development, the temperature and moisture ranged of 18-25 ºC and 50-90 %, respectively. The rainfalls ranged of 0-20 mm, with exception of the wheat plant emergency initial stage, which had until 55 mm of rainfall. These data were collected from the National Institute of Meteorology (*INMET*, 2013).

2.2.4 Plant Development

The wheat plant were grown until anthesis stages (about 70 days after planting) in the soil of the experimental field area. In this stage, when the individuals showed completed spikelet development, the plants were transferred from soil to pots of 30 x 60 cm (height x width) containing the same soil of the field experimental area. A number of 20 wheat plants in this growth stage were transferred to each pot. This were taken to the Plant Growth Chamber, where received *Fusarium* inoculation and Zn treatment and maintained in the pots until maturation stage (final phase) into a with temperature (25 ± 1.4 ºC) and moisture (66 ± 6.8 %) controlled during the whole period.

2.2.5 Plant - *Fusarium* inoculation and Zn Treatment

The plants (n = 20 in triplicates, total = 60 plants per treatment) were divided into Groups: *Controls* - I (no *F. graminearum* and Zn-treatment), II (with addition of $ZnSO_4$), III (with addition of ZnO-NPs), IV (only *F. graminearum*) and *Treated* - V (*F. graminearum* and $ZnSO_4$) and VI (*F. graminearum* and ZnO-NPs). *(a) Fusarium inoculation:* all the Groups IV, V and VI received 5 mL suspension of *F. graminearum* (10^6 conidia/mL) by spray in each spikelet flowered in the anthesis stage. The Control Group received only the sterile solution (0.8 % NaCl). *(b) Zn-treatment:* the Treated Groups V and VI received after the fungal inoculation, 5 mL of the solution of $ZnSO_4$ (100 mM) and ZnO-NPs (100 mM) by spray in each spikelet flowered and the Control Group IV only 5 mL of sterile solution (0.8 % NaCl). Next, the plants were maintained in the pots under light control, irrigation, moisture and temperature until the maturation phase (about 90 days after planting). Physiological wheat maturity stage is usually defined as the time when the flag leaf and spikes turn yellow (*Hanft* and *Wych*, 1982). The Fig. 2 show the maturation phase with spikelet complete, presenting the pinkish color with signs of FHB caused by *F. graminearum.*

2.2.6 Samples Harvesting (wheat and husks)

At the maturation phase, each whole spikelet was collected and the mature wheat grains, manually harvested. They were taken into sterile polyethylene bags and identified for subsequent analysis: humidity (a_w and mc), mycology, DON levels, SEM observation and Zn residues. See flowchart of the whole experiment in Fig. 3.

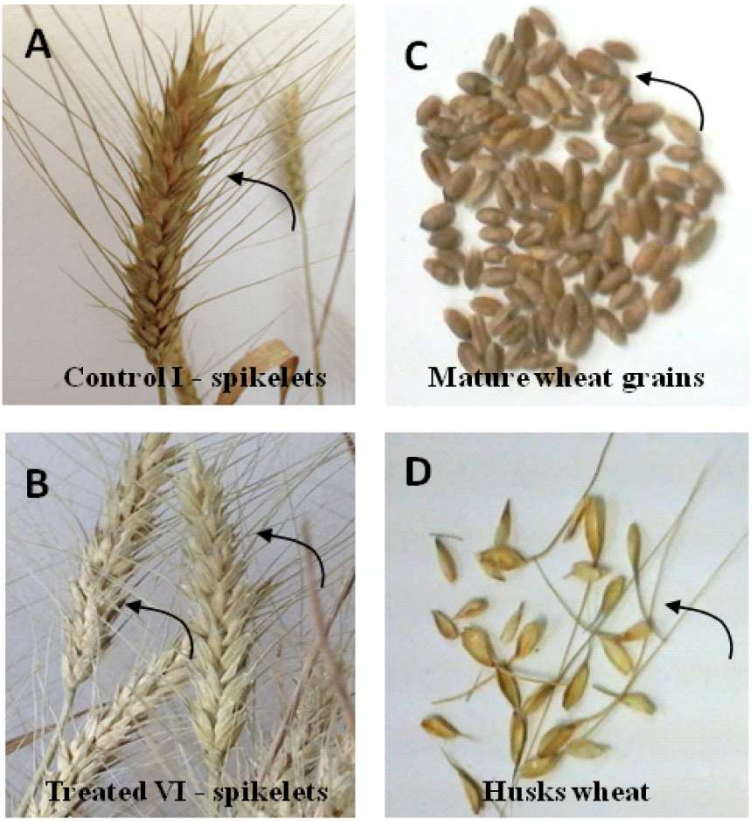


Figure 2: Wheat plant at maturation phase (ca. 90 days after planting). (A) Plant Control I (without the addition of any *F. graminearum* and Zn-compound) and (B) Plant Treated VI (with *F. graminearum* and ZnO-NPs) in the pots; (C) mature wheat grains and (D) husks freshly harvested.

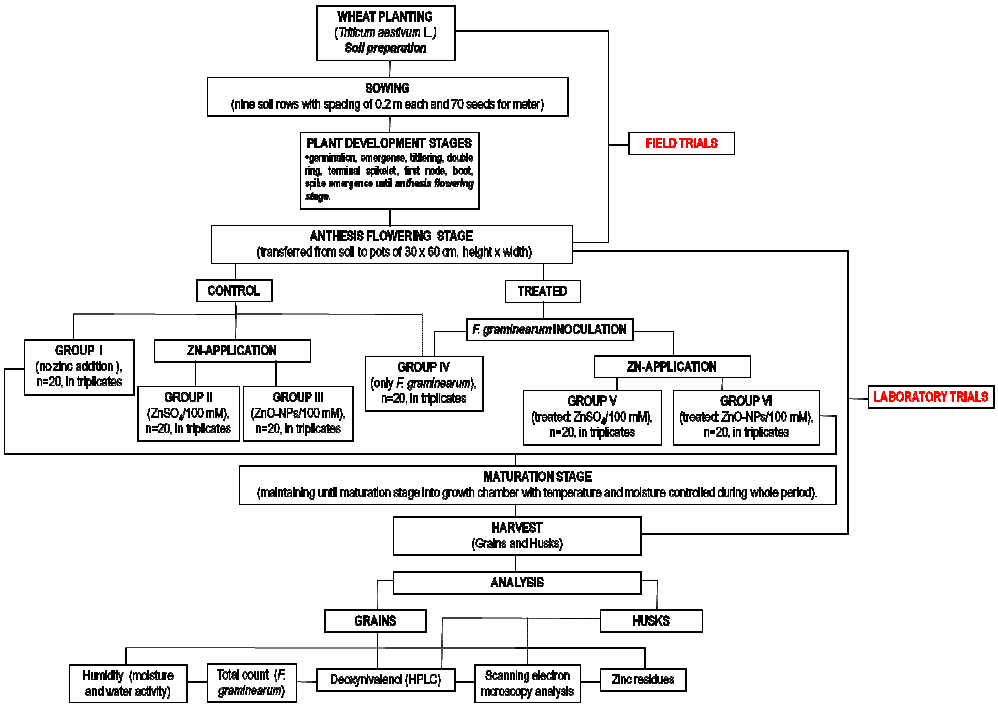


Figure 3: Chart flow of the whole experiment, showed wheat plant development and treatment, followed of subsequent analysis in the harvested wheat grains.

2.2.7 Humidity and Mycological Tests (wheat grains)

In order to define mc, wheat grains (2 g) were submitted to a drying process in an oven (105°C ± 5°C) until a constant weight through gravimetric measures (*AOAC*, 2005). In addition, wheat grains (2 g) were submitted to Aqua-Lab 4TE equipment for a_w determination. All analyses were performed in triplicate. With respect to the mycological analysis, the colonies enumeration technique was applied to evaluate the total fungi load (*Silva* et al., 2010). Wheat grains (10 g) collected from each artificially contaminated plant sample were added to 90 mL of 0.1 % peptone dissolved in water under sterile conditions. The mixture was stirred in a rotary shaker for 2 min and the dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained. Aliquots of 0.1 mL of each dilution were spread (in duplicate) over a surface of the PDA medium containing chloramphenicol (100 mg l^{-1}) and incubated up to 7 days at 28 ºC in the dark. The results were expressed in colony forming units per gram (CFU g^{-1}).

2.2.8 DON Quantification (wheat grains and husks)

Whole wheat grains and husks samples were analyzed using IACs for the cleaning step and LC/UV for detection, according to the DON Test protocol G1005 (*Vicam*, 2013), with some modifications. Briefly, 10 g of grains or husks samples were ground in a laboratory mill and transferred to an industrial blender jar with 50 and 100 mL of LC grade water, respectively. The mixture was blended for 30 seconds, followed by filtration and cleaned using the IACs. The filtrate sample (5 mL) was loaded and eluted at a flow rate of one drop per second using the vacuum manifold. After washing the column, the toxin was slowly eluted with 100 % LC grade methanol. The eluate was concentrated using a heating block device (at 40 ºC) with gentle nitrogen stream and the dry residue redissolved in mobile phase (acetonitrile:water - 10:90, v/v). The extract (20 µL) was injected into the LC/UV System set at a wavelength equal to 218 nm and the mobile phase delivered at a constant flow rate of 0.6 mL/min. The DON method validation was carried out through measurements of peak area at DON retention time compared with the standard solutions used for calibration curve (0.15, 0.20, 0.25, 0.5, 1, 2, 3, 4, 5, 7.5, 10 and 15 µg ml^{-1}), reaching a correlation (*r*) of 0.996. The recovery process was set by spiking DON-free

wheat samples to get DON concentrations of 250, 1000 and 1500 $\mu g\ kg^{-1}$ on the same day and at the same HPLC conditions.

2.2.9 Scanning Electron Microscopy Analysis (wheat grains and husks)

The wheat grains and husks presenting pink color were selected for analysis in SEM in order to observed *F. graminearum* contamination. Others grains of the Groups Control and Treated grains were randomly selected for the observation of the pericarp grain. The grains were put into aluminum stubs using metal adhesive glue and placed on the Au Coater holder. Afterwards, vacuum (up to 10^4 mBar) was applied and they were coated with a 1.40 nm Au layer. Next, a SEM microscope analysis of the stubs were made after being submitted to the vacuum process. Thus, they were identified taking into account the different magnifications and recorded as micrographies (voltage from 0.5 to 30 kV).

2.2.10 Zn Residues (wheat grains)

Grains (20 g) after grinding were analyzed for possible Zn residues by dry ashing in muffle, followed ash acid dilution and Zn content was determined by atomic absorption spectrophotometry according to analytical methods (985.35) described by *AOAC* (2005).

2.2.11 Statistical Analysis

All data were analyzed taking into account the analysis of variance (ANOVA) and, additionally, considering the Tukey Multiple Comparison Test. The results were expressed in mean ± standard deviation and values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Effects of Zn-compounds Treatments on Humidity of Freshly Harvested Wheat Grains (mc & a_w)

Regarding the mc levels, it was observed a significant difference between the Controls IV (only *F. graminearum*) and Treated V and VI (*F. graminearum* and Zn-compounds application - $ZnSO_4$ and ZnO-NPs), with mc of 11.94, 12.41 and

12.56 %, respectively. In contrast, the analysis of a_w revealed that only the Control Group IV was statistically different, with less a_w registered (0.5284) when compared to Control Group I (no *F. graminearum* and Zn), a_w 0.5561 (Table 1).

3.2 Effects of Zn-compounds Treatments on *F. graminearum* and DON of Freshly Harvested Wheat Grains and Husks

3.2.1 *F. graminearum* growth

The analyses revealed no significant difference on fungi colonies growth between the Control Groups (I, II and III), with low fungal colony content (1.1 - 1.3 x 10^1 CFU g^{-1}) (Table 1). In contrast, among the Treated Groups (V and VI) with *F. graminearum* and Zn-compounds, only the Treated VI (ZnO-NPs) was statistically different (5.8 x10^2 CFU g^{-1}) when compared to Control IV (only *F. graminearum* - 2.8 x10^3 CFU g^{-1}), which represent a 79.28% growth reduction and therefore, highest efficiency. In addition, the Treated V (with $ZnSO_4$) did not showed statically reduction of *F. graminearum* growth.

3.2.2 DON formation

Regarding the validation of the DON method applied, its retention time was 17 ± 0.5 min with calibration curve being linear from 0.15 to 15 µg/mL, correlation (*r)* of 0.996. The LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) was of 66.7 and 119.1 µg kg^{-1}. The recovery experiments showed yields of 87 ± 9, 96 ± 6 and 93 ± 3 % considering concentrations of 250, 1000 and 1500 µg kg^{-1}, respectively. Thus, the recovery mean of the extraction method was quite good reaching 92 ± 4 % which allowed us to proceed the DON measurements. As expected, the Control Group *F. graminearum* inoculated (no Zn treated), presented the highest DON levels on the grains (Control IV: 1063.22 µg kg^{-1}) (Table 1). On the other hand, the Treated VI (ZnO-NPs) showed the best results considering *F. graminearum* contamination, which there was not DON formation in the harvested wheat grains, showing antimycotoxigenic efficiency of this treatment. Already the Treated V ($ZnSO_4$) had been not efficient on *F. graminearum* inhibition in this grains, nevertheless, the DON concentration was slightly reduced (721.16 µg kg^{-1}) in relation to Control IV (1063.22 µg kg^{-1}), which represent reduction of 32.17 %. The same occurred in

grains husks, that presented high DON levels in Control IV (1970.98 µg kg^{-1}), however in this case, DON was detected in Treated V ($ZnSO_4$) and VI (ZnO-NPs), which there was presence of 409.70 µg kg^{-1} and 615.56 µg kg^{-1} and represents 79.21 % and 68.77 % of DON levels reduction. As expected, DON mycotoxin levels in harvested wheat grains and husks were not detected between the Controls Groups (I, II and III), which no received *F. graminearum* inoculation.

3.3 Zn-compounds Treatments Effects on Microstructures of Freshly Harvested Wheat Grain and Husks

The microstructure observed by SEM did not present any visible difference on the grains pericarp (extern & intern surfaces) morphology between the Treated Groups V and VI (Fig. 4.A.2 and Fig. 4.A.3) when compared to Control Groups I and IV (Fig. 4.A and 4.A.1). Additionally the microstructure characteristics of grains structure did not demonstrate any apparent damage (deformations and/or ruptures) caused by the chemical agents. However, in the Control Group (IV) that received only *F. graminearum* (Figs. 4.A.1, B) the fungal hyphae and conidia were present the on grains pericarp external surface. More details were possible to observe in micrographs images at x1.500 magnification (Fig. 4.B.1). Equally, in the husks the presence of fungal hyphae and conidia were evident and in higher amounts than the grains pericarp (Fig. 4.B.2). More details can to be observed in Fig. 4.B.3.

3.4 Zn-compounds Treatments Effects on Zn Residue of Freshly Harvested Wheat Grain

As expected, the Control Group (IV), which did not receive Zn treatment, showed quite low levels of that element in the harvested wheat grains (mean: 31.05 mg kg^{-1}). In contrast, the Treated Groups V and VI, that received $ZnSO_4$ and ZnO-NPs treatments, had higher Zn levels with 55.74 and 41.98 mg kg^{-1}, respectively, which represents a Zn residue increase of 79.51 and 35.20 % in the freshly harvested wheat grain. Table 2 show the wheat grains Zn residue *versus* daily intakes (DI) recommended by FDA (2011), ODS (2011) and FAO (2002).

Table 1: Effects of zinc-compounds treatments during wheat cultivation on harvested wheat grains (moisture content, water activity, *Fusarium* growth and deoxynivalenol formation).

Groups[b]	Wheat Plant			Replicate (n)	Wheat Grains[a]			DON (µg kg^{-1})	
	Fusarium inoculation	Zn-compound application			mc (%)	a_w	*F. graminearum* (CFU g^{-1})	Grains	Husks*
		Treatment	Compound (particle size)						
Controls									
I	NA[c]	NA	NA (NA)	3	12.71 (±0.0141)	0.5561 (±0.0234)	1.3 x 10^1 (±0.3 x 10^1)	ND[e]	ND
II	NA	A[d]	$ZnSO_4$ (regular)	3	12.45 (±0.0494)a	0.5381 (±0.0005)	1.3 x 10^1 (±0.1 x 10^1)	ND	ND
III	NA	A	ZnO (nano)	3	12.48 (±0.0919)a	0.5384 (±0.0004)	1.1 x 10^1 (±0.2 x 10^1)	ND	ND
IV	A	NA	NA (NA)	3	11.94 (±0.0212)a	0.5284 (±0.0006)a	2.8 x 10^3 (±1.4 x 10^2)a	1063.22(±14.79)a	1970.98 (±72.11)a
Treated									
V	A	A	$ZnSO_4$(regular)	3	12.41 (±0.0070)a,b	0.5545 (±0.0034)	2.6 x 10^3 (±2.1x10^2)a	721.16 (±1.68)a,b	409.70 (±5.10)a,b
VI	A	A	ZnO (nano)	3	12.56 (±0.0212)a,b	0.5427 (±0.0015)	5.8 x 10^2 (±0.7x10^1)a,b	ND b	615.56 (±2.63)a,b

[a] values are expressed as mean ± SD; sample collection of plant maturation (about 90 days of cultivation)
[b] Groups: Control I (no *F. graminearum* and Zn), II (only $ZnSO_4$/100 mM); III (only ZnO-NPs/100 mM); IV (only *F. graminearum*) and Treated V (*F. graminearum* and $ZnSO_4$/100 mM); VI (*F. graminearum* and ZnO-NPs/100 mM) (n = 20 per Group, in triplicates). [c] not applicable / [d] applicable / [e] ND - no detected
a - statistically significant when compared to Control Group - I ($p<0.05$) by Tukey Multiple Comparison Test.
b - statistically significant when compared to Control Group - IV ($p<0.05$) by Tukey Multiple Comparison Test.

Table 2: Effect of Zn-compounds Treatments on Zn Residual Concentration in the Harvested Wheat Grain and tolerable maximum consumption

Wheat Plant Zn treatment		Wheat grains Zn residue (mg kg^{-1})	Zn recommended DI (mg day^{-1})			Wheat grains Zn treated consumption (kg day^{-1}) (to reach TDI mg day^{-1})	
Groups	Concentration applied		Minimum[a]	TDI[b]			
			FDA	FDA/ODS	FAO/WHO	FDA/ODS	FAO/WHO
No Zn		31.05				1.29 (40.05)	1.45 (45.02)
$ZnSO_4$	100mM[c]	55.74	11 and 8	40	45	0.72 (40.13)	0.81 (45.15)
ZnO-NPs		41.98				0.95 (39.88)	1.07 (44.92)

[a]Total recommended amount to daily intake (DI) adults is 11 and 8 mg day^{-1}, for men and women respectively.
[b]TDI: Tolerable daily intake.
[c] applied during plant anthesis stage (on spikelets: grains formation)

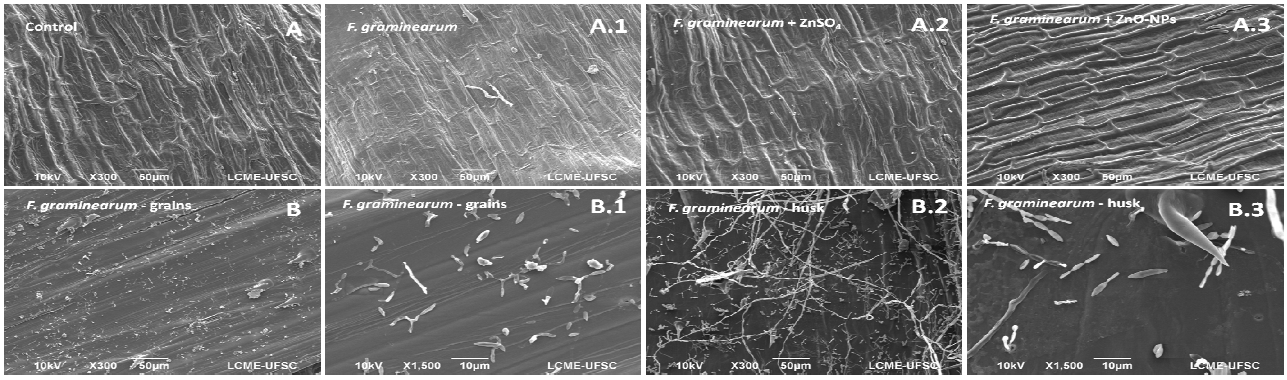


Figure 4: Scanning electron microscopy (SEM) images [300 to 1500x] of microstructure characteristic of wheat grain (A-A.3, B, B.1) and husk surface (B.2, B.3). Details of external surface of the grains pericarp represents (A) Control Group I (without *F. graminearum* and no addition of any Zn-compounds); (A.1) Control Group IV (only with *F. graminearum* inoculated); (A.2) Treated Group V (with *F. graminearum* inoculation and $ZnSO_4$/100 mM application) and (A.3) Treated Group VI (with *F. graminearum* inoculation and ZnO-NPs/100 mM application); (B,B.1) details of *F. graminearum* contamination in the external surface of the grains pericarp and (B.2,B.3) *F. graminearum* contamination in the external surface of the grains husks. SEM micrographs images in magnification of x300 (A-A.3, B, B.2) and in more details in x1500 (B.1, B.3).

4 Discussion

The wheat plant is most susceptible to FHB by *F. graminearum* during anthesis, therefore, in general, the experimental inoculations are carried out at the flowering stage. In most practical breeding programs, a spore suspension is sprayed on the wheat heads. Varieties and breeding lines may be grouped for earliness. FHB ratings are determined either, as the product of the percentage of heads infected and the proportion of infected (bleached) spikelets, of three to four weeks after inoculation. In the presence of moisture the spores germinate, hyphae enter via the cracked anthers and grow down the anther filament into the host plant, especially because the wheat anthers are one of the most important entry pathway for *F. graminearum* to produce FHB (*McMullen* et al., 1997).

Generally on the field, high temperatures (25 to 35 ºC) and a_w (0.70 to 0.90) are favorable conditions for the *F. graminearum* growth and DON formation in the wheat plant spikelet at anthesis stage. Despite the *F. graminearum* artificial inoculation in the Control (IV) and Treated Groups (V, VI), there was little variations of mc (11.94-12.56 %) and a_w (0.5284-0.5545) in the wheat grains and the values meet the required Brazilian regulation limits (*Brazil*, 2010).

New antifungal agents that can assist in the current control strategies are necessary to avoid FHB in wheat. In laboratory experiments, there are only few studies that report the antifungal activity of Zn-compounds (*Stoimenov* et al., 2002; *He* et al., 2011; *Savi* et al., 2013a; *Savi* et al., 2013b). These studies had been performed only in laboratory trials, not checking toxigenicity in the grains though. *Sharma* et al. (2011) found strong antifungal activity of ZnO-NPs (10 mM) synthesized by microwave method against the plant fungus (*Pythium debarynum*). *He* et al. (2011) showed that 12 mM of ZnO-NPs was enough to completely inhibit *Botrytis cinerea* and *P. expansum* growth (not focusing on toxin production).

In studies carried recently by our laboratory, *Savi* et al. (2013a) found that the colony diameter growth of *F. graminearum* was significantly reduced by ZnO (23 mm) and ZnO-NPs (30 mm) when compared with Control (60 mm), being completely inhibited by $ZnSO_4$. Regarding DON formation, authors reported that *F. graminearum* strain only produced the toxin when grown on ZnO medium treated. On the other hand, ZnO (at nano size) and $ZnSO_4$ (at regular size) inhibited fungal growth and their ability to produce DON (*Savi* et al., 2013a). In addition, all tested fungi in this studies showing combined effects on conidia reduction, hyphae alterations, mortality and ROS formation due

to Zn-compounds treatments. Therefore, our study has been conducted to evaluate antifungal and antimycotoxin activities of Zn-compounds on wheat plant, in order to avoid the DON formation in the grains. The Zn-NPs treatment was the best on *F. graminearum* growth and DON formation, this compound in nano size, seems to have penetrated in wheat husks reaching and protecting the grains with highest efficiency.

In the other hand, in field experiments, several Biological and Chemical Control studies have been reported on wheat plants in order to avoid FHB by *F. graminearum*. Biological Control, such as bacteria (*Lysobacter enzymogenes*, *Bacillus amyloliquefaciens*, *Microbacterium oleovorans*, *Enterobacter hormaechei*, *Bacillus subtilis*, *Brevibacillus sp.*) and fungi (*Clonostachys rosea* and *Cryptococcus flavescens*) (*Xue* et al., 2014). Regarding the Chemical Control, such as synthetic pesticides application (insecticide and fungicide), studies have investigated their efficacy on *Fusarium* and mycotoxins formation in maize (*Folcher* et al., 2009; *De Curtis* et al., 2011) and in all results obtained, the efficacy was greater when the insecticide was applied in combination to fungicide. *De Curtis* et al. (2011) found that, the treatment applied alone, reduced FBs content in the grain only in fifty of the cases, whereas fungicide treatments applied in combination with the insecticide showed a highest significant reduction of FBs contamination. On the other hand, *Folcher* et al. (2009) found a reduction on trichothecenes group level of around 73.5 % with insecticide and a higher reduction of 84.2 % with insecticide plus fungicide. However, the levels of contamination of fungi trichothecenes producing were just 7.6 and 5.4 % for insecticide and insecticide plus fungicide treatment, respectively. In that case, the treatments were not statically different, showing efficiency only to reduce the mycotoxin level but not the fungi infection. In the same occurred in our current study by applying $ZnSO_4$ treatment, which showed to be efficient to DON reduction whereas the fungi growth remained similar to the Control. However, ZnO-NPs treatment was efficient in both cases, reducing totally the DON formation.

The microstructure observed by SEM did not present any visible difference caused by the chemical agents on the grains pericarp (such as, deformations and/or ruptures). Instead, the Zn amount significantly increased after the treatment with $ZnSO_4$ and ZnO-NPs when compared to Controls. However, the increase of Zn in wheat grains can to be nutritionally advantageous, due to its characteristics as an essential mineral for the human body in physiological and metabolic processes of several tissues and organs formation, especially to the

immune system. Moreover, it can be utilized as dietary supplement, already authorized for the fortification of foods (*FDA*, 2011; *ODS*, 2011; *FAO*, 2002). In addition, Zn is an essential micronutrient for plants which is recommended as fertilizer for crops.

Several studies have been published regarding the use of Zn for food fortification and recommended amounts (*Tripathi* et al., 2010; *Bautista-Gallego* et al., 2013). Therefore, Zn-compounds applications as antifungal must agree with tolerable upper limit for human consumption, since that Zn residues may remain in food. The tolerable upper intake of Zn depends of consumer life stage. For adults, the recommended amount is 11 and 8 mg day^{-1}, for men and women respectively. However the tolerable DI (TDI) for adults is up to 40 and 45 mg day^{-1} recommended by FDA (2011)/ODS (2011) and FAO/WHO (2002), respectively.

For some scientists, there is a need to increase the Zn concentration and its bioavailability in wheat grain, especially in wheat flour (the most often and high consumed product) and represents an urgent challenge. Furthermore, the wheat processing after harvest substantially reduces the concentration of Zn and also other minerals, which further increases the Zn deficiency in humans (*Zhang* et al., 2010).

Khoshgoftarmanesh et al. (2013) observed that the addition of Zn in the form of $ZnSO_4$ by spray application on foliar at the early anthesis stage, increased significantly Zn grains content (ca. 40 mg kg^{-1}) of most wheat cultivars.

In our study, the Zn-compounds application in the wheat plant increased Zn residual in the grains, this content accords with the mentioned studies to increase the Zn concentration in wheat grains and then not reach the upper limit of human daily consumption (*FDA*, 2011; *ODS*, 2011; *FAO*, 2002).

5 Conclusion

This is the first study showing the efficiency of chemical control of FHB by Zn-compounds both (ZnSO4 and ZnO-NPs) in wheat plant cultivation, with the best treatment obtained with ZnO at NPs size. That compound efficiently prevented *F. graminearum* growth and DON formation in the grains at low concentration. Besides, Zn remained within international recommended level of Zn for consumption and the treatment did not developed any damage to wheat grains. New strategies of control using Zn-compounds in addition to conventional treatment

could increase the efficiency against *F. graminearum* growth and DON formation. Furthermore, studies are required to identify specific combinations between chemical treatments in order to avoid the FHB in the field wheat plant. Nevertheless, for studies performed totally in the field (at all plant development stages) the grains quality must be taken into account.

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9 CAPÍTULO 7
Efeito do Gás Ozônio sobre a Exposição de Espécies Fúngicas Toxigênicas dos Gêneros *Fusarium, Aspergillus* e *Penicillium*

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Effects of Ozone Gas Exposure on Toxigenic Fungi Species from *Fusarium, Aspergillus,* and *Penicillium* Genera

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Abstract

The antifungal properties of ozone (O_3) gas exposure towards toxigenic fungi were evaluated in laboratory scale treatments trials. O_3 gas was utilized at a concentration of 60 µmol/mol at different times of exposure, which efficiently inhibited the fungi colonies growth, especially *F. graminearum* and *P. citrinum*. At the concentration applied, O_3 gas exposure was able to inhibit conidia germination, caused hyphae morphological alterations that led to hyphae death and ROS production of all fungi tested. O_3 action can be related to cell metabolism alterations, leading to apoptosis and oxidative stress, showing to be effective on controlling toxigenic fungal development which is one of the main problems regarding food contamination.

Keywords Ozone Gas, Fungi, Conidia, Hyphae, Mortality, Oxidative Stress

INTRODUCTION

Fungi are often found on the field and during food storage, however the production of mycotoxins depends on environmental conditions, upon the fungal species, as well as composition of the commodity, and so the conditions of harvesting, handling and storage (Bryden 2009). Those are responsible for raw and processed grains deterioration. Some species can cause plant pathologies leading to seed/grain loss of germination, discoloration and reduction of nutritional values. Especially, when exposed to optimal environment conditions as high temperature and humidity, some species can produce mycotoxins in food and grains. The fungal species most often reported contaminating raw materials and processed food belong primarily to five genera: *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* (Brase et al. 2009). The genera of most globally concern are

Fusarium, *Aspergillus*, and *Penicillium*, especially *Fusarium* sp. genera in the field crops and *Aspergillus* and *Penicillium* sp. during grain storage (Llorens et al. 2004; Scussel et al. 2011a). The major toxins produced by these three genera include: aflatoxins, ochratoxin A, citrinin, trichothecenes, fumonisins, and zearalenone (Scussel 2002), that can cause acute or chronic intoxication and damage to humans and animals health after contaminated food ingestion (Marasas and Nelson 1987; Moss 1996).

These fungi can be controlled by the application of synthetic fungicides on the field or during storage. However, fungicide treatment has several disadvantages due to its high toxicity to mammals and the possibility to leave residuals, which may remain in food products (Barlow 1985; Boobis et al. 2008). The ozone (O_3) gas application as decontamina-tion agent may have several advantages in the food industry. The attractive aspect of O_3 gas is that it decomposes to molecular oxygen at ambient temperature without leaving residues (USFDA 2005). In 1982, the U.S. Food and Drug Administration (FDA) classified O_3 for treating bottled water as *generally recognized as safe* (GRAS) (USFDA 1982). Moreover, the potent disinfectant characteristics of O_3 are recognized by the Food and Agriculture Organization (FAO 1994).

O_3 is a powerful antimicrobial agent due to its poten-tial oxidizing capacity (Khadre et al. 2001). It is currently used as disinfectant towards microorganisms and viruses, for odor removal and for removing taste, color, and decomposi-tion of organic matter (Cataldo 2008; Karaca and Velioglu 2009; Karaca et al. 2010). O_3 has been effectively used to control fungal growth at laboratory-scale trials in food, as bar-ley, wheat, fig and Brazil nuts (Kottapalli 2005; Scussel et al. 2011b; Wu et al. 2006; Zorlugenç et al. 2008) and reduce mycotoxin contamination in peanut, fig, Brazil nuts and in field trials for artificially contaminated corn (Chen et al., 2014; McDonough et al. 2011; Scussel et al. 2011b; Zorlugenç et al. 2008). These studies were carried out to investigate the efficiency of O_3 treatment in the preservation of foods. Nevertheless little is known about the direct effect of O_3 exposure on fungal survival and development (Antony-Babu and Singleton 2009, 2011; Tzortzakis et al. 2008) and to our knowledge no research study has examined the effect of O_3 exposure on toxigenic fungi species.

The objective of this research project was to explore the *in vitro* antifungal effects of O_3 gas on different fungi strains from *Fusarium*, *Aspergillus*, and *Penicillium* genera where colony growth, conidia germination, and hyphae alterations (morphology, mortality and reactive

oxygen species (ROS) production) were highlighted by applying light microscopy (LM), scanning electron microscopy (SEM), and fluorescence microscopy (FM).

MATERIALS AND METHODS

Materials

Fungi Strain

Fusarium graminearum, *F. verticillioides*, *Penicillium citrinum*, *Aspergillus parasiticus*, and *A. flavus* obtained from the Food Mycology Laboratory of Mycotoxicology and Food Contaminants (LABMICO) culture collection at the Federal University of Santa Catarina, Florianopolis, SC, Brazil.

Equipment

The following equipment for microscopy was used: light microscope (LM), Olympus (Shinjuku, Tokyo, Japan); scan-ning electron microscope (SEM), JEOL (Peabody, MA, USA); fluorescence microscope (FM), Leica (Leider, IL, EUA). Others equipment were an O_3 generator, model OP-35-5L, Interzon (Jundiai, SP, Brazil); impurities remover, Ouro Peças (Alvorada, RS, Brazil); flow meter, Protec (São Paulo, SP, Brazil); critical point dryer, model CPD 7501, Quorum Technologies (Guelph, Canada); gold (Au) coater, model SCD500, Leica (Leider, IL, USA); autoclave, Phoenix (Araraquara, SP, Brazil); colonies counter, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); ultra-violet cabinet, Dist (Florianopolis, SC, Brazil); Eppendorf centrifuge model 5415R, Eppendorf (São Paulo, SP, Brazil); microbiological incubator, Quimis (Diadema, SP, Brazil); vacuum pump, Tecnal (Piracicaba, SP, Brazil).

Chemicals

The reagents were sodium chloride and potassium from Vetec (Duque de Caxias, RJ, Brazil) and formaldehyde 37%, Tween 80, ethyl alcohol, sulphuric acid, sodium thiosulfate, potassium iodine from Synth (Diadema, SP, Brazil). The dyes utilized were 2,7-

dichlorohydrofluorescein diacetate (H_2DCF-DA) and Evans blue dye of the Sigma Aldrich (St Louis, MO, USA) and lactophenol cotton blue from Newprov (Pinhais, PR, Brazil); the culture media was potato dextrose agar (PDA) of the Sigma Aldrich (St. Louis, MO, USA).

Methods

Ozone Gas Preparation

The O_3 gas generated was applied to five chambers with capacity of 21 × 14 cm (length × diameter). Four chambers were exposed for 40, 60, 90, and 120 min to O_3 gas and the fifth one to control treatment conditions (room air, no ozone) in triplicates. The chambers were made of sealed cylindrical transparent glass with only two apertures: one for the input of O_3 gas and one for the output. The O_3 gas generator system followed the procedures detailed Giordano et al. (2012) with minor modifications, as described along with this section: the compressed air pump was connected to an air impurities remover to filter the room air. The impurities removed were solid particles and humidity. After the filtering of air was con-ducted, the air flow meter was adjusted to 1 L/min and then the O_3 generator was calibrated to reach a concentration of 60 µmol/mol. The O_3 generator utilized (5–60 µmol/mol) was of corona discharge type process where O_3 is produced by electrical discharge when air or pure oxygen passes between the two electrodes. The O_3 gas produced was introduced through a tube into the input aperture of each chamber. The Control chamber (without O_3 gas) was ventilated with "room air" in the same flow (1 L/min). The O_3 gas concentration measurement was performed by the iodinemetric test. (a) Iodinemetric test method: O_3 concentration was measured in each chamber by the titration method on the outlet of the O_3 generator. The gas was bubbled into a potassium iodide solution (50 mL), acidified with 2.5 mL of sulfuric acid 1 N (pH below 2.0). The solution was then titrated with sodium thiosulfate 0.005 N using a starch solution as indicator, according to APHA (1999).

Ozone Gas Application and Its Effects on Fungi

A disc (4 mm) with *F. graminearum, F. verticillioides, P. citrinum, A. parasiticus* and *A. flavus* mycelia material and conidia, taken from the edge of 7-days-old-fungal culture was placed individually inside the chamber at a height of 4 cm above the bottom

surface of the chamber.

(a) Fungi growth inhibiting treatment: The fungi was treated in the chamber with an O_3 gas concentration of 60 µmol⁄mol and exposed for 40, 60, 90, and 120 min, whereas the fungi in the Control received room air at the same exposure times. Afterward, treated fungi from all chambers including the Control were placed on each Petri dish center containing culture medium PDA and incubated at 25 ºC for 8 days. The efficiency of O_3 gas treatment was evaluated until the 8th day after incubation by measuring the fungi colony diameter (in mm) (Fraternale et al. 2003; Savi et al. 2012).

(b) Conidia germination: to evaluate the conidia germination, fungi were treated with O_3 gas with a concentration of 60 µmol⁄mol for 120 min. The treated fungi colonies were transferred to a 0.89% NaCl and 0.1% Tween 80 solution tube and stirred for fungi conidia detachment. The conidia solution (100 µL) was placed on the surface of a microscope slide containing culture medium PDA. This slide was transferred into a sterile Petri dish containing moist cotton and incubated for 28 ºC in the dark for 15 h.After, a drop of lactophe-nol cotton blue was placed on the growth medium to stop the conidia germination and to perform the plate counting (Marques et al. 2004). Finally, 100 conidia were counted in each area of the microscope slide, utilizing LM at 400×.

(c) Hyphae alterations: morphology, mortality and ROS production: the five fungi strains (mycelia and conidia) received O_3 gas treatment with the concentration of 60 µmol⁄mol for 90 min to evaluate possible hyphae morphology alterations, on the other hand for mortality and ROS production, the exposure time was 120 min. The Control hyphae (without O_3 gas exposure) received room air flow, with the same conditions as the treated ones.

(c.1) Morphology: to verify morphological alterations in the fungi treated with O_3 gas, SEM analysis were performed. Treated and Control fungi sections were collected, fixed with formaldehyde to preserve fungal structures, washed with phosphate buffer saline (PBS) and dehydrated with alcohol solution (30, 60, 80, 90, and 100% and keeping longer the mycelia at 100%) then, it was taken for critical point drying. Afterwards, fungi mycelia were prepared for SEM analysis as follows: *Fungi Strain Stubs and Gold (Au)-Coating Preparation:* Strains were fixed onto stubs (diameter 1.2 mm, height 0.8 mm), placed on the Au Coater holder, applied vacuum (up to 10^4 mBar) and coated with a

1.40 nm Au layer; *The Fungi Mycelia SEM Observation:* Stubs with Au-coated fungi strains were transferred to SEM microscope, submitted again to vacuum and fungi morphology visualized, identified at different magnifications and registered by micrographies (taken at a voltage of 0.5−30 kV) (Bray 2000).

(c.2) Mortality: the treated and Control hyphae were soaked in 0.05% Evans blue dye solution and left to stand for 5 min. After, the hyphae were washed three times with PBS to remove the excess of dye. The resultant sediment containing treated fungal hyphae was analyzed in LM at 400× magnification. The hyphae were observed according to their staining differences (Evans blue staining: dead hyphae and natural color: intact hyphae) (Semighini and Harris 2010). In each area of the microscope slide, 100 fungal hyphae (dead or live) were counted.

(c.3) ROS production: the treated and Control hyphae were treated with 40 µM H_2DCF-DA for 30 min at 28 ºC in the dark. The sediment was washed (3×) with PBS and analyzed by FM in 300× magnification. The ROS produc-tion was observed by coloration of green fluorescent hyphae (Semighini and Harris 2010). The presence of fluorescence of the fungal hyphae were observed in each area of the microscope slide.

Statistical Analysis:

The data of fungi colonies growth, conidia germination and hyphae mortality were analyzed by analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were expressed as average ± standard deviation and the *p*-values < 0.05 were considered statistically significant.

RESULTS

Effects of Ozone Gas on Fungi Growth

The 60 µmol/mol O_3 gas treated fungi strains at the exper-iment longest treatment time (120 min) had the most reduced colony growth (Figure 1). The strain of *F. graminearum* that received O_3 gas exposure for 60 min did not present growth until the 8th day of incubation. For the strain of this fungi that received O_3 gas exposure for 40 min, the growth was significantly smaller (38 mm) when compared to the Control (53 mm) at the end of the incubation. Also *P. citrinum* was not able to grow on culture medium PDA after receiving 120 min of O_3 gas

exposure.

Furthermore, in the other times of O_3 gas exposure (40, 60, and 90 min), its growth was significantly smaller (19, 16, and 10 mm) than the Control (23 mm). On the other hand, *F. verticillioides*, *A. parasiticus*, and *A. flavus* had similar behaviors among them. There were resistant to treatment and growth on culture medium after O_3 gas exposure in different times (40, 60, 90, and 120 min). Nevertheless, their growth was significantly smaller after 120 min of O_3 gas exposure (52, 63, 60 mm) when compared with control (63, 88, 78 mm), respectively.

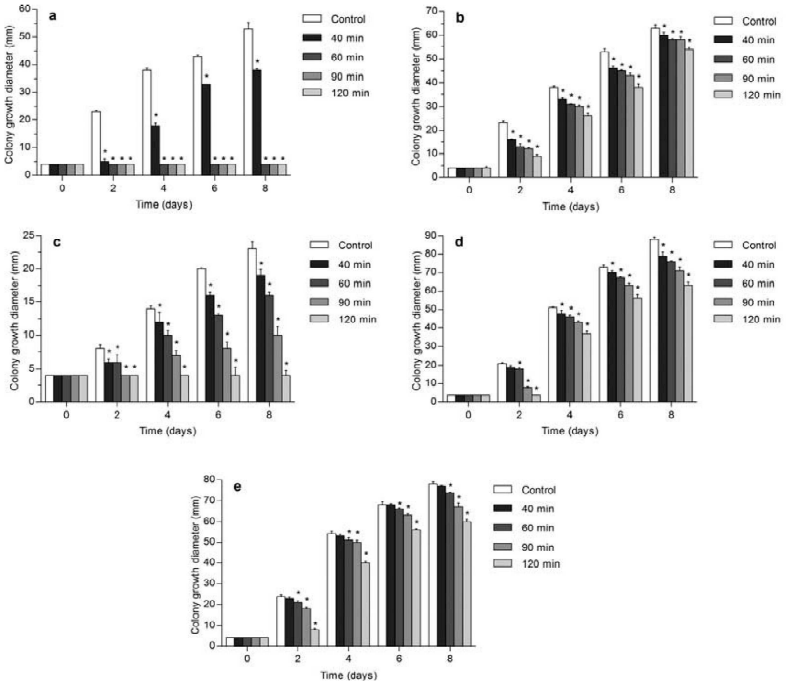

FIGURE 1. Effects of ozone gas (60 µmol/mol, 40/60/90/120 min of exposure) on different fungi genera and species growth: (a) *F. graminearum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *A. parasiticus*, and (e) *A. flavus* (data are shown as fungi diameter average values and standard deviation). Symbols indicate statistically significant when compared with control group * $p < 0.05$.

Effect of Ozone Gas on Conidia Germination

After incubation for 15 h at 28 ◦C, conidia germination was strongly inhibited by the O3 gas treatment when compared with the Control (Figure 2). Therefore the most effective conidia germination inhibition was observed at the longest period of O3 gas exposure (120 min). Under this treatment, the reduction of *F. verticillioides*, *A. parasiticus*, *A. flavus*, *P. citrinum*, and *F. graminearum* conidia germination were 39, 27, 17, 9, and 3%, respectively, when compared to Control (80, 96, 84, 98, and 99%, respectively).

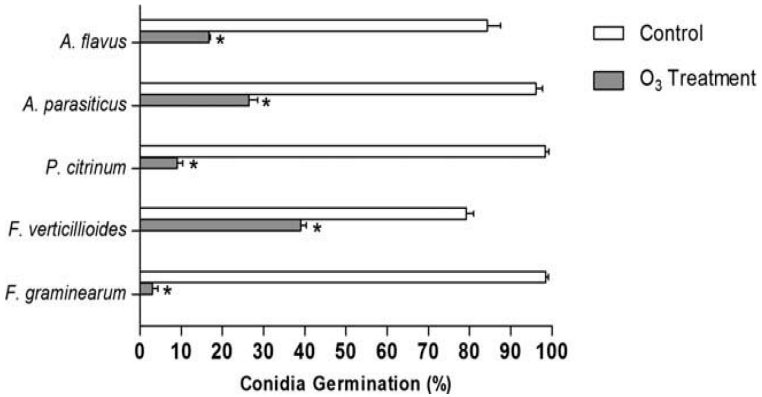

FIGURE 2. Effects of ozone gas (60 µmol/mol, 120 min exposure) on fungi conidia germination. Data are shown as average values and standard deviation of conidia germination percentage. Symbols indicate statistically significant when compared to Control group *$p < 0.05$.

Effect of Ozone Gas on Hyphae Alterations: Morphology, Mortality and ROS Production

Morphology: all fungi showed deformations and rup-tures in the hyphae structure. In addition, after treatment was not verified the presence of perfect conidia, such as was found in the Control (Figure 3). The O_3 gas exposure caused morphological changes during the formation of fungi structure (conidia and hyphae), possible resulting in ruptures of the fungal cell membrane and growth reduction.

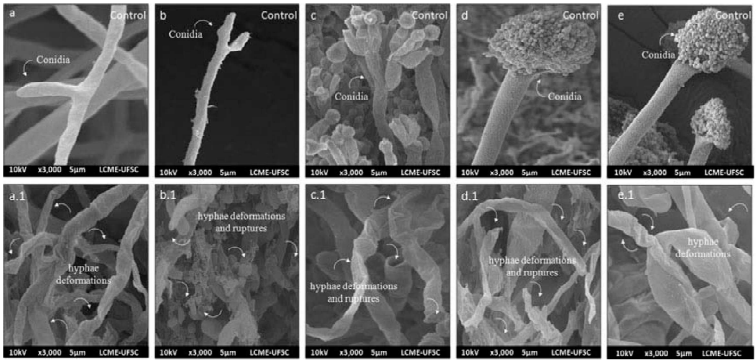

FIGURE 3. Effects of ozone gas (60 µmol/mol, 90 min exposure) on hyphae morphology of (a) *F. graminearum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *A. parasiticus* and (e) *A. flavus*. [*Control*: no treatment (a,b,c,d,e); *Ozone gas treatment:* (a.1, b.1, c.1, d.1 and e.1), showing hyphae alterations]. SEM micrographs images, x3,000.

Mortality: the O_3 gas treatment was effective for hyphae mortality in the concentration of 60 µmol/mol treated for 120 min. The percentage of hyphae mortality after O_3 gas exposure was the highest in *F. graminearum* (97%) and *P. citrinum* (96%), followed by *F. verticillioides* (77%), *A. flavus* (51%), and *A. parasiticus* (49%). In the Control strains, the percentage of hyphae mortality were very low for *F. graminearum* (1%) and *A. parasiticus* (3%), followed by *A. flavus* (4%), *P. citrinum* (5%), and *F. verticillioides* (7%) (Figure 4a). The O_3 gas treatment lead to cell death, verified by Evans blue staining distribution in the fungi hyphae as shown in Figure 4b. All treated fungi showed blue staining after the O_3 gas treatment suggesting the presence of dying hyphae causing failure of fungi cell integrity. These hyphae were unable to exclude the dye, therefore remaining stained deep blue. However, healthy Control hyphae were able to exclude Evans blue dye and, therefore keeping intact cell integrity, showing their natural color.

ROS production: the treated fungi hyphae with O_3 gas showed an increase in the ROS production. All fungi after treatment showed a strong intensity green fluorescence inside the hyphae structure due to the intracellular ROS formation (Figure 5). Control hyphae showed no ROS production due to the lack of observed fluorescence. The ROS production verified in the treated hyphae may be related to a chemical stress caused by O_3 gas exposure, which was expected since as

mentioned earlier, O_3 is a strong oxidant reagent.

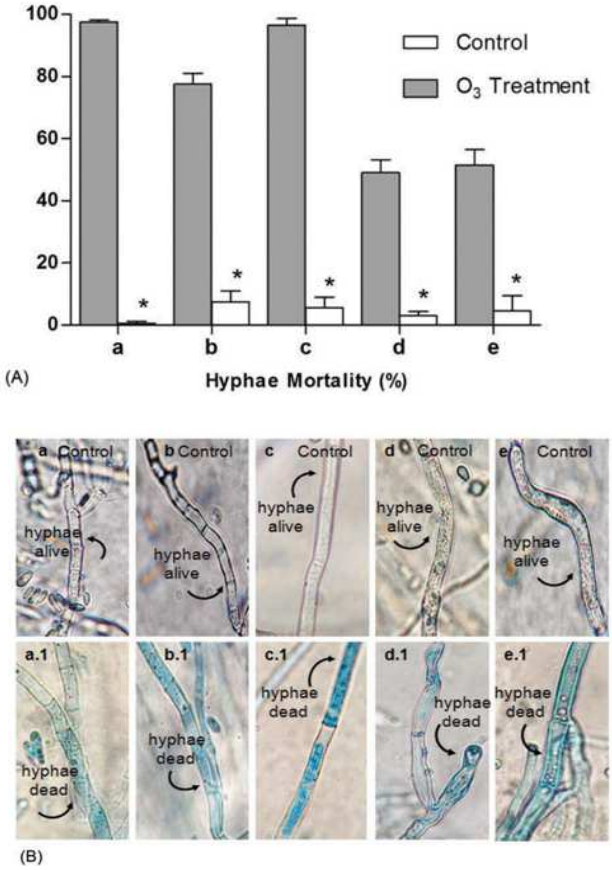

FIGURE 4. Hyphae mortality of the ozone gas treated fungi (60 µmol/mol, 120 min exposure): (A) showing data as average and standard deviation (%) and (B) Evans blue stain distribution: (a) *F. graminearum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *A. parasiticus*, and (e) *A. flavus* [*Control*: no treatment - without coloration (hyphae alive - a,b,c,d,e); *Ozone gas treatment:* hyphae coloration (hyphae dead - a.1, b.1, c.1, d.1, e.1), showing hyphae alterations]. LM images at x400.

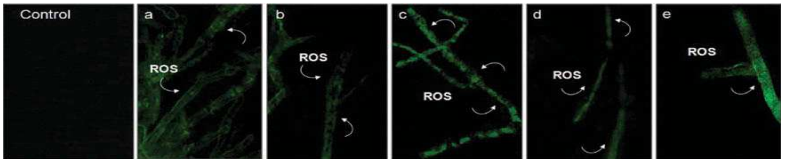

FIGURE 5. Effect of the 2,7-dichlorohydrofluorescein diacetate on ozone gas treated fungi (60 µmol/mol, 120 min exposure): (a) *F. gramin-earum*, (b) *F. verticillioides*, (c) *P. citrinum*, (d) *A. parasiticus*, and (e) *A. flavus* [Control: no treatment (no ROS production in all fungi); *ozone gas treatment:* hyphae fluorescence development (after ROS production - a, b, c, d, e)]. FM images, x300.

DISCUSSION

Strategies of decontamination with O_3 gas application has been recommended for food preservation and destruction of microflora (Inan et al. 2007; Keutgen and Pawelzik 2008; Najafi and Khodaparast 2009; Selma et al. 2008). Studies in the literature showed that O_3 gas has been effectively used to control fungal growth in laboratory scale trials in food and grains (Giordano et al. 2012; Kottapalli et al. 2005; Scussel et al. 2011b; Wu et al. 2006; Zorlugenç et al. 2008). However, there are only few studies that report the mechanism of action of the O_3 gas exposure on filamentous fungi (Antony-Babu and Singleton 2009, 2011; Ozkan et al. 2011) and none on the toxigenic species that was utilized in this research project. The effect of the O_3 gas direct exposure on fungi morphological alterations and cell death is still not understood.

In this research, the resistance of the treated fungi to O_3 varied. O_3 gas was efficient in inhibiting especially of *F. graminearum* and *P. citrinum*. They were inhibited completely by the treatment (60 µmol/mol) for 120 min exposure. An O_3 level higher than 60 µmol/mol would be required to achieve complete fungi death. However, in food application, one O_3 concentration should be used up to a level that does not cause biochemical changes, which will depend upon the chemi-cal food composition, time and the concentration of O_3 gas applied, apart from the type of application (Cárdenas et al. 2011).

On the other hand, for *A. ochraceus*, visual growth was not seen until the end of 5 days for all treatments, and after 7 days no growth differences were seen between O_3 exposed and Control cultures. The same authors, in 2011, also studied the effect of O_3 treatment on growth of a *Eurotium amstelo-dami* IS-SAB-01 species, isolated from naan bread, utilizing two O_3 treatments a low-level, long-term exposure (0.4

µmol/mol for 21 days) and high-level, short-term exposure (300 µmol/mol for 5 to 120 min). For the low-level exposure the combination of different media sucrose concentrations (0, 5, 10, and 20% w/v) with O_3 treatment was also assessed. The growth of the isolate was found to be sensitive to low-level O_3 treatment depending on the media sucrose concentration and duration of the exposure (Antony-Babu and Singleton 2011). The O_3 gas applications forms in the previous research were different from our study; however, it demonstrated efficiency in the fungi growth inhibition.

When fungus is exposed to extreme adverse conditions of growth (i.e., chemical treatments, low temperature, and high humidity) including long term storage (Aregger 1992; Savi et al. 2013), the number of conidia germination can be reduced. This occurred in the current research, especially with *F. graminearum* and *P. citrinum*, as the conidia germination were significantly inhibited by the O_3 gas exposure, followed of *A. flavus, A. parasiticus,* and *F. verticillioides*.

Very low concentrations of O_3 (0.3−1.5 µmol/mol) caused inhibition of the mycelial growth and sporulation of several fungi in food, including *B. cinerea* and *Sclerotinia sclerotiorum* on strawberries (Nadas et al. 2003) and grapes (Palou et al. 2002), *P. digitatum* on citrus fruit (Palou et al. 2001) and tangerine fruit (Boonkorn et al. 2012), and *Rhizopus stolonifer* on table grapes (Sarig et al. 1996). Antony-Babu and Singleton (2011) found that high-level of O_3 exposure (300 µmol/mol) for short exposure times (5 to 120 min) reduced *Eurotium* spore viability, although 100% reduction in viability was achieved only after 120 min exposure. As conidia germination reflects the fungi development capacity, our data corroborates especially for *F. graminearum* and *P. citrinum*, which showed the highest sensibility against both fungi growth and conidia germination reduction.

In addition to reduce fungi reproductive capacity, the chemical treatments can induce cellular damage typical of O_3, such as the induction of cell death (Faoro and Iriti 2009; Heath 2008). In the current research, the O_3 gas exposure lead to hyphae cell death of all fungi tested and consequently fungi growth reduction in different proportions. *F. graminearum* and *P. citrinum* may be more sensitive to treatment related to fungi colony growth, conidia germination, and hyphae mortality, than *F. verticillioides, A. parasiticus,* and *A. flavus*. The fungi cell death can occur due to metabolism changes and general oxidative stress, for example, the increase intracellular ROS formation.

ROS formation plays an important role on the healthy aerobic cells signaling and homeostasis. Its production typically occurs at a

controlled rate, however under chemical stress, it can be greatly increased. This behavior is responsible for fungi cell death due to apoptotic-like cell and nucleic acids mutations/carcinogenesis production. In cell plant, death cell and ROS production were verified due to O_3 gas exposure (Faoro and Iriti 2005; Langebartels et al. 2002; Overmyer et al. 2003; Pasqualini et al. 2003; Rao and Davis 2001).

In this research, all fungi showed ROS increased forma-tion with O_3 gas exposure when compared to the Control. Hence, it is likely that the cellular response to O_3 induced oxidative stress could have caused a collapse in intracellular redox balance and thus affecting the fungi cell developmental processes. This is the first study that showed what occurred with fungi cells after O_3 gas exposure in terms of conidia germination, fungi hyphae morphology, mortality, and ROS production and the resistance of each fungi tested.

CONCLUSION

All fungi were significantly affected by O_3 gas treat-ment application. The best results of antifungal effect were observed on *F. graminearum* and *P. citrinum*, that were completely inhibited, followed of *F. verticillioides*, *A. parasiticus*, and *A. flavus* that were more resistant to O_3 gas. This is the first study where the effect of O_3 treatment on toxigenic fungi was described, including the hyphae and conidia mor-tality, morphology modifications of hyphae, and the induction of ROS on them caused by O_3.

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10 CAPÍTULO 8

Eficiência do Tratamento com gás ozônio frente ao crescimento de *F. graminearum* & degradação de deoxinivalenol nos grãos de trigo integral (*Triticum aestivum* L.) e seus efeitos sobre a qualidade e germinação

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Ozone treatment efficiency on *Fusarium graminearum* and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum* L.) quality and germination

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Abstract

The contamination of wheat grains by *Fusarium graminearum* is directly associated with deoxynivalenol (DON) production. In order to overcome fungal growth, ozone gas (O_3) has been widely used in food laboratory trials, but the application of O_3 at high doses may affect grain quality attributes. The objective of this study was to assess the efficacy of O_3 treatment on wheat grains artificially contaminated by *F. graminearum* and DON. In addition, several physical and biochemical properties after treatment were investigated. The tests were performed in laboratory pilot cylinders containing grains, divided into control groups (no O_3 gas) and treated groups (40 and 60 mmol/mol), which were exposed for 30, 60, 120 and 180 min. After 30 min of O_3 exposure, *F. graminearum* showed significant growth reduction at both concentrations, complete inhibition occurring after 180 min. With regard to mycotoxins, the DON levels of pericarp and endosperm grain were depleted and after 120 min of exposure, DON was eliminated from both grain portions. Afterward, physical and biochemical analysis showed that there was a significant difference between the carboxyl contents of control and treated wheat starches only after 180 min of O_3 exposure. X-ray diffraction (XRD), lipid peroxidation, total protein profile and scanning electron microscopy (SEM) did not reflect any significant differences after O_3 treatment. The application of O_3 treatment only affected wheat germination after 180 min of exposure, reducing germination capacity by 12.5%. Additionally, there were no changes with respect to the length of the coleoptile or seminal root of germinated wheat seeds. In summary, O_3 gas was effective against fungal growth and caused DON degradation, especially after 120 min at 60 µmol/mol concentration, without causing physical and biochemical

changes in whole wheat grains.

Keywords: *Fusarium graminearum*; Deoxynivalenol; Whole wheat grains; Ozone; Quality.

1. Introduction

Fusarium species, especially *Fusarium graminearum*, cause Fusarium Head Blight (FHB) disease in wheat. This disease occurs primarily through an inoculum of *F. graminearum* [teleomorph: *Giberella zeae* (Scw.) Petch] ascopores, which are produced in fungal perithecia on wheat crop residues. The spores are carried by wind over long distances and deposit in the anthers of wheat during the flowering period, infecting the plant and causing depigmentation of affected spikelets (white to pinkish) (Panisson et al., 2002; Lima, 2004).

Among the trichothecene mycotoxins associated with FHB, deoxynivalenol (DON) isthe most common in wheat grains (Muthomi et al., 2008; Bensassi et al., 2010; Soleimany et al., 2012; Stankovic et al., 2012; Ennouari et al., 2013; Santos et al., 2013). Human and animal exposure to DON through the ingestion of contaminated food can induce acute and chronic effects such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity (Rotter et al., 1996; Wijnands and Van Leusden, 2000; Pestka, 2007).

Since 2012, federal regulation in Brazil has set the maximum tolerable levels (MTLs) for DON in whole wheat grains at 2000 µg/kg. The limit will be decreased over time to allow grain producers and the industry to adapt to the legislation without causing a shortage of wheat. As of January 2014, DON limits for whole wheat grain have been set at 1500 µg/kg, and in January 2016, they will be at 1000 µg/kg (Brazil, 2011). The lower limit of DON set by the European Communities Commission is 1250 µg/kg for unprocessed cereals in general and 1750 µg/kg for unprocessed durum wheat (European Commission, 2006).

To reduce fungal growth and various mycotoxins in wheat grains while mitigating post-harvest losses, safe and effective technoloies are increasingly needed. One of these new technologies is the ozonation of food products. Ozone (O_3) is a powerful antimicrobial agent due to its potential oxidizing capacity (Khadre et al., 2001). It is currently used as a disinfectant for microorganisms and viruses, odor and taste removal, color and decomposition of organic matter (Cataldo, 2008; Karaca and Velioglu, 2009; Karaca et al., 2010). O_3 has been effectively used to control growth of various fungi in laboratory-scale

trials in food, such as barley, wheat, figs and Brazil nuts (Kottapalli et al., 2005; Wu et al., 2006; Zorlugenç et al., 2008; Scussel et al., 2011), to reduce mycotoxin contamination in peanuts, figs and Brazil nuts, and in field trials for artificially contaminated corn (Dwarakanath et al., 1968; Zorlugenç et al., 2008; McDonough et al., 2011; Scussel et al., 2011). The US Food and Drug Administration (FDA) declared O_3 safe for bottled water in the United States and as a secondary direct food additive (FDA, 1982). Gaseous O_3 treatments are used in fruit storage to reduce the buildup of volatiles such as ethylene, which promote unwanted ripening (Skog and Chu, 2001).

Application of O_3 in doses that are sufficient for the effective decontamination of grains may affect quality in several ways. O_3 gas is not universally beneficial and in some cases can promote oxidation and/or degradation of chemical constituents present in the grains. Starch and lipid oxidation, protein modifications, grain discoloration or seed germination loss may occur from excessive use of O_3 gas (Tiwari et al., 2010).

The objective of this study is to evaluate the efficacy of O_3 treatment on *F. graminearum* and DON levels in artificially contaminated wheat grains and, in addition, investigate the effect of O_3 on several physical and biochemical properties, namely oxidation and X-ray diffraction (XRD) of isolated wheat starch, lipid peroxidation, total protein profile and scanning electron microscopy (SEM) analysis of wheat grains and seed germination.

2. Materials and methods

2.1. Materials

2.1.1. Culture media

Potato dextrose agar (PDA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil).

2.1.2. Chemicals

DON standard, sodium hydrogen sulphite, hydroxylamine, sodium dodecyl sulfate, 2-mercaptoethanol, glycerol, bromophenol blue and coomassie brilliant blue were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA); acetonitrile, methanol, chloramphenicol, acetic acid, hydrochloric acid, sodium hydroxide,

trichloroacetic acid, thiobarbituric acid were obtained from Vetec (Duque de Caxias, RJ, Brazil), all of LC grade. High-purity Milli-Q water (18.2 MΩ/cm) was obtained from a Millipore Synergy system (MA, USA). Other materials: immunoaffinity columns from DON-Test Vicam (Milford, MA, USA).

2.2. Instruments

For the mycological tests, the following equipment was required: (i) light microscopes (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); (ii) autoclave, Phoenix (Araraquara, SP, Brazil); (iii) microwave oven, Philco (Sao Paulo, SP, Brazil); (iv) laminar flow cabinet, Veco (Campinas, SP, Brazil); (v) fume cabinet, Quimis (Diadema, SP, Brazil); (vi) rotary shaker, Marconi (Piracicaba, SP, Brazil); and (vii) microbiological incubator, Quimis (Diadema, SP, Brazil). To analyze the moisture content (mc) and water activity (aw) the following equipment was used: a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil) and Aqua-Lab 4TE Decagon Devices (Sao Jose dos Campos, SP, Brazil), respectively.

The whole wheat grain samples were ground in a Romer 1301 (Union, MO, USA) laboratory mill. The determination of DON levels was carried out by a high performance liquid chromatograph (HPLC) model 321, Gilson (Middleton, WI, USA) equipped with an isocratic pump model 805, manual injector (20 µL loop) and with ultravioletevisible (UV) detector model 118 set at 218 nm. The chromatographic column used was a C18 250 x 4.60 mm reversed-phase, Synergi 4 mm particle size Fusion-RP 80, Phenomenex (Madrid Avenue, Torrance, USA).

The following equipment was required for physical and biochemical tests: (a) laboratory blender, Skimsen (Brusque, SC, Brazil), (b) laboratory mill Romer, (c) centrifuge, Nova Tecnica (Piracicaba, SP, Brazil), (d) laboratory water bath, Dubnoff (Florianopolis, SC, Brazil), (e) pH meter, Quimis (Diadema, SP, Brazil), (f) magnetic stirrer, Ikamag (Campinas, SP, Brazil), (g) X-Ray diffractor Cade-4, Enraf-Nonius (Eugene, OR, USA), (h) electrophoresis apparatus and power supply from Bio-Rad (Hercules, CA, USA), (i) spectrophotometer, Biospectro (Curitiba, PR, Brazil) and (j) scan-ning electron microscope (SEM), Jeol (Peabody, MA, USA).

2.3. Samples

About 50 kg of wheat grains were collected from vertical silos

(stored for three months post-harvest) in 2012, from the Brazilian Agricultural Research Corporation (Embrapa Wheat). No partic-ular preference was used to select the samples. Samples were received after cleaning and drying (up to a maximum of 60 ºC) in the storage unit, packed in a polyethylene bag and stored at 4 ºC for analysis at the Laboratory of Mycology, Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Cata-rina, Florianopolis, Santa Catarina, Brazil.

2.4. Samples artificial contamination

To accomplish the mycological analysis, a solution (5 mL) of Tween 80 containing 1 x 10^4 spores/mL of *F. graminearum* were sprayed on wheat grains (25 g) (mc =16%). To find the mc, wheat grains (2 g) were dried in an oven (105 ºC ± 5 ºC) to a constant weight using the gravimetric method. To perform the mycotoxin analyses, firstly the wheat grains (7 g) were spiked using 50 µL of DON standard solution (200 µg/mL) in order to produce a grain pericarp contamination. Secondly, 50 µL of the same DON solution was injected with a syringe and needle into the grain endosperm (mc = 14%). For the physical and biochemical analyses after O_3 treatment, the grains (mc = 12%) were not artificially contaminated. The grains were conditioned at a temperature of 25 ± 0.5 ºC. All these analyses were performed in triplicate and according to the Association of Official Analytical Chemists - AOAC (2005) guidelines.

2.5. Ozone gas treatment in the storage pilot cylinders

The laboratory pilot cylinders used were 25 cm x 10 cm (length x diameter) with two apertures: one for the input of O_3 gas (bottom) and one for the output (top). The cylinders were filled with 350 g of wheat grains. At the top (above grains), a polyamide screen surface was placed in order to support the grains on which would be performed the mycological (25 g), mycotoxin (7 g), physical and biochemical analyses (25 g) after O_3 gas application.

The O_3 gas was briefly applied to pilot cylinders, divided into control (no O_3 gas) and treated groups (40 µmol/mol and 60 µmol/mol). They were exposed for 30 min, 60 min, 120 min and 180 min, in a room

at 25 ± 0.5 ºC.

For the mycological analysis, wheat grains were exposed to 40 and 60 µmol/mol for 30, 60, 120 and 180 min. For the mycotoxin analysis, they were only exposed to 60 µmol/mol for 30, 60 and 120 min. Based on these analyses, the most effective concentration against the *F. graminearum* and DON (60 µmol/mol for 120 and 180 min) was chosen for further physical and biochemical analysis.

The O_3 gas generator system followed the procedures detailed by Giordano et al. (2012) with minor modifications. First, the compressed air pump was connected to a device responsible for clearing the air impurities and, consequently, getting rid of solid particles and humidity. Afterward, the filtered air was driven to the adjusted flow meter at 1 L/min and then the O_3 generator was calibrated to reach a concentration of 40 or 60 µmol/mol.

The O_3 production by the generator (5-60 µmol/mol) used the corona discharge process, in which an electrical discharge caused by the passage of air or pure oxygen (O_2) between the two elec-trodes generates the conversion of O_2 to O_3. Thus, the O_3 gas pro-duced was injected through a tube into the input aperture of each test chamber while the control chambers were ventilated with "room air" at the same flow rate (1 L/min).

The O_3 gas concentration was measured by the iodinemetric titration test from the output of the O_3 generator. The gas was bubbled into potassium iodide solution (50 mL) acidified with 2.5 mL of sulfuric acid 1 N (pH below 2.0) and titrated with sodium thiosulfate (0.005 N) using a starch solution as indicator, according to APHA (1999).

2.6. Mycological analysis after ozone treatment

The enumeration technique was applied to evaluate the total fungi load (Silva et al., 2010). Twenty five grams of each artificially contaminated wheat sample were added to 225 mL of 0.1% peptone dissolved in water under sterile conditions. The mixture was stirred in a rotary shaker for 2 min and dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained. Aliquots of 0.1 mL of each dilution were spread (in duplicate) over the surface of PDA medium plates containing 100 mg/L chloramphenicol and incubated for up 7 days at 28 ºC in the dark. The results were expressed in colony forming units per gram (CFU/g).

2.7. DON analysis after ozone treatment

Whole wheat grains samples were analyzed using immu-

noaffinity columns for the cleaning step and LC/UV for detection, according to Vicam protocol DON Test, N_o G1005 USA (Vicam, 2013), with some modifications. In summary, 7 g of each artifi-cially contaminated wheat sample were ground in a laboratory mill and placed in an industrial blender jar with 30 mL of LC grade water. The mixture was blended for 30 s, followed by filtration and cleaning using an immunoaffinity column (DONTest HPLC). This column was first conditioned with 1 mL of LC grade water after which the filtrate sample (1 mL) was added at a flow rate of one drop per second.

After washing the column with 2.5 mL of LC grade water, the toxin was slowly eluted with 2 mL of 100% LC grade methanol. The eluate was evaporated using a heating block device at 40 ºC in a gentle nitrogen stream and thereby the dry residue was redissolved in 100 mL of mobile phase acetonitrile:water (10:90, v/v). The extract (20 µL) was injected into the LC/UV System set at wavelength 218 nm and the mobile phase was delivered at a constant flow rate of 0.6 mL/min.

To obtain the DON level scores, the measurement of peak area at DON retention time was compared with the standard solutions used for calibration curve (0.150, 0.200, 0.250, 0.5, 1, 2, 3, 4, 5, 7.5, 10 and 15 µg/mL) with a correlation r = 0.996. The recovery process was set by spiking DON-free samples of wheat with DON concen-trations of 250, 1000 and 1500 µg/kg on the same day and at the same HPLC conditions.

2.8. Physical and biochemical analysis of wheat grains after ozone treatment

2.8.1. Starch isolation

The starch was isolated from wheat grain groups (control and treated) using the procedure of Rupollo et al. (2010) and Vanier et al. (2012), taking into account that 250 g of wheat grains were ground using a laboratory mill. Subsequently, the flour obtained was added to distilled water containing 0.16% sodium hydrogen sulphite to break down the protein/starch matrix and to prevent microbial growth (Singh et al., 2004). After 24 h at 4 ºC, the water was drained off and the slurry was mixed in a laboratory blender and sifted using a 200-mesh sieve. The material remaining in the sieve was washed thoroughly with distilled water and the filtrate slurry was allowed to stand for 3 h after which the supernatant was removed and the settled starch layer was re-suspended in distilled water and centrifuged at 1200 g for 20 min. The upper non-white layer was scraped off. The white layer was then re-

suspended in distilled water and centrifuged at 1200 g for 15 min. The upper non-white layer was scraped off once again, and the starch was collected and dried in an oven at 40 ºC for 12 h.

2.8.2. Carbonyl content

The carbonyl content of the isolated starch obtained from the wheat grain was determined according to the titrimetric method described by Smith (1967) and Vanier et al. (2012). Two gram of the starch sample was placed in distilled water (100 mL) in a 500 mL flask. The suspension was gelatinized in a boiling water bath over 20 min, subsequently cooled to 40 ºC and then the pH level was set at 3.2 with 0.1 N HCl. A hydroxylamine reagent (15 mL) was added to the mixture and the flask was sealed and placed in a 40 ºC water bath for 4 h with slow stirring. The excess hydroxylamine was determined by titration by means of a reaction with standardized HCl and pH set at 3.2.

Similarly, a blank experiment with native wheat starch and hydroxylamine reagent was performed. In this case, 20 mL of hydroxylamine reagent (25 g of hydroxylamine hydrochloride in 100 mL of 0.5 N NaOH and made up to 500 mL with distilled water) was added. The carbonyl content was expressed as the quantity of carbonyl groups per 100 glucose units (COOH/100 GU), calculated as described by Smith (1967) and Vanier et al. (2012).

2.8.3. Carboxyl content

The carboxyl content of the isolated starch was determined according to the modified procedure of Chattopadhyay et al. (1997) and Vanier et al. (2012). Approximately 2 g of a starch sample was mixed with 25 mL of 0.1 N HCl, and the slurry was stirred occa-sionally for 30 min. Next, the slurry was vacuum-filtered using a 150 mL medium porosity fritted glass funnel and washed with 400 mL of distilled water. The starch was carefully moved to a 500 mL beaker, and the volume was made up to 300 mL with distilled water.

Once this process was concluded, the starch slurry was heated in a boiling water bath with continuous stirring for 15 min and then made up to 450 mL with more distilled water. It was then titrated to pH 8.3 with standardized 0.01 N NaOH.

Native wheat starch was used as blank sample of the determination. The carboxyl content was expressed as the quantity of carboxyl groups per 100 glucose units (COOH/100 GU), calculated as described by Chattopadhyay et al. (1997) and Vanier et al. (2012).

2.8.4. XRD

X-ray diffractograms of the isolated starches were obtained with an X-Ray diffractor according to Sandhu et al. (2012). The scanning region of the diffraction ranged from 5 º to 35 º with a target voltage of 30 kV, current of 10 mA, 0.06 of angular increment and time interval of 1 s.

2.8.5. Lipid peroxidation

Lipid peroxidation was determined according to Dhinsa et al. (1981) and Michalowicz et al. (2009). In this study, 0.5 g of the control wheat sample and 0.5 g of the treated grain were mixed with 5 mL of 0.1% trichloroacetic acid (TCA). After centrifuging at 10,000 rpm for 10 min at 4 ºC and the supernatant was reduced to 1 mL, 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA was then added and the solution was heated to 96 ºC for 30 min and then cooled in ice to return to room temperature. Lipid peroxidation was calculated by absorption value at 532 nm, using the molecular extinction coefficient of 155 mM^{-1} cm^{-1} for malonaldialdehyde, a thiobarbituric acid reactive substance (TBARS).

2.8.6. Protein analysis

SDS-PAGE was performed according to Li et al. (2012), using 10% of separation gel (pH 8.8) and 5% stacking gel (pH 6.8). For each analysis, 50 mg of control wheat and 50 mg of treated wheat were each ground in a mill and stirred in 1 mL of extraction buffer (0.01 M TriseHCl, pH 6.8, including 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol (2-ME), 10% (v/v) glycerol, 0.1% (w/v) bromphenol blue). For non-reduced proteins, extraction proceeded in a buffer without 2-ME.

All samples were heated for 5 min at 100 ºC and then centrifuged for 5 min at 8000 g. Aliquots of 15 mL were loaded into each well and electrophoresis was performed at 120 V. The gel was stained with 0.25% w/v Coomassie brilliant blue and de-stained in 10% acetic acid.

2.8.7. SEM analysis

The wheat grains groups (Control and Treated) and the isolated starch were put into aluminum stubs using metal adhesive glue and placed on the Au Coater holder. Afterward, vacuum (up to 10^4 mBar) was applied and they were coated with a 1.40 nm layer of gold.

Next, a SEM microscope analysis of the stubs was made after submission to the vacuum process. Thus, they were identified taking into account the magnification rates and recorded as micrographies (voltage from 0.5 to 30 kV).

2.9. Seed germination

The wheat seed germination was examined before and after O_3 treatment according to the method proposed by the International Seed Testing Association (1985). The seeds were allowed to germinate between two blotter paper layers at 25-27 ºC for 8 days and the percentage germination was calculated. The tests were repeated four times and the averages were recorded.

2.10. Statistical analysis

All data were analyzed taking into account the analysis of variance (ANOVA) and, additionally, considering the Tukey or Bonferroni as post-tests. Thus, the main results were expressed in terms of mean ± standard deviation and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mycological analysis after ozone treatment

The viability of *F. graminearum* significantly reduced (12.5 ± 10^1 and 4 ± 10^1 UFC/g, respectively, at concentrations of 40 and 60 µmol/mol) after 30 min of O_3 exposure when compared to the control group (48 ± 10^1 UFC/g), which represents a 74.5 and 91.8% reduction of viable spores. In turn, after 180 min of O_3 exposure at these concentrations, no growth of *F. graminearum* occurred on the test plates (Fig. 1).

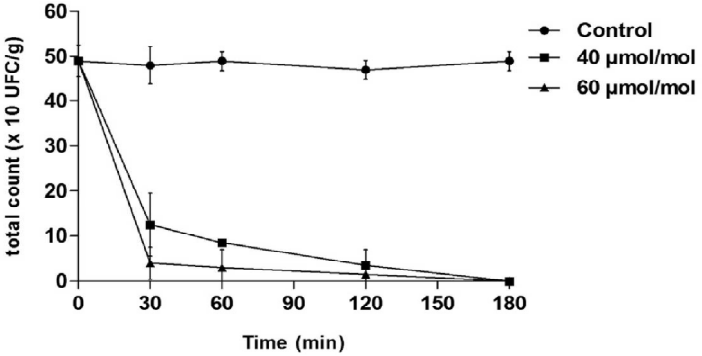

Fig. 1. Ozone gas effects (40 and 60 µmol/mol in 30-180 min of exposure) on viability of *F. graminearum* (data are shown as total count). All treatments were statistically significant when compared to the control group ($p < 0.05$) by Bonferroni post-test.

3.2. DON analysis after ozone treatment

The LC/UV method for DON chromatographic separation and the validation parameters obtained (linearity, limit of detection e LOD, limit of quantification e LOQ, reproducibility, repeatability and recovery), were quite adequate. Under the chromatographic con-ditions used, the DON retention time (Rt) was equal to 17 ± 0.5 min.

Linearity was confirmed using the calibration curve for each DON concentration; i.e., it was linear from 0.15 to 15 µg/mL (correlation coefficient 0.996). The LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) was 0.067 and 0.12 µg/g (67 and 119 µg/kg), respectively.

The recovery experiments showed yields of 87 ± 9%, 96 ± 6% and 93 ± 3% for concentrations of 250, 1000 and 1500 µg/kg, respectively, a mean recovery rate for the extraction method of 92 ± 4%.

In pericarp grain, after 30, 60 and 120 min at 60 µmol/mol O_3, DON levels (205.9, 123.7 µg/kg and no detection) were significantly reduced when compared to the control group (1065, 923 and 981 µg/kg). The same effect occurred in the endosperm grain, as shown in Table 1, although magnitudes were different. In summary, the DON levels decreased as they were exposed to O_3 treatment.

Based on these results, it is apparent that the O_3 gas has a greater impact on the external part of the wheat grain in the elimination of DON than in the endosperm.

Table 1. Degradation of deoxynivalenol (μg/kg) by ozone gas (60 μmol/mol for 30, 60 and 120 min of exposure).

Wheat grain			O_3 gas		DON
Group	n[a]	Part of grain	Concentration (μmol/mol)	Exposure time (min)	Average (μg/kg)
Control	3	Pericarp	NA[b]	30	1065.10
				60	923.13
				120	980.90
Treated	3	Pericarp	60	30	205.91*
				60	123.71*
				120	ND[c]*
Control	3	Endosperm	NA	30	535.88
				60	472.12
				120	436.15
Treated	3	Endosperm	60	30	424.73
				60	311.71*
				120	ND*

* Symbols indicate statistically significant differences when compared with control group $p < 0.05$ by Bonferroni post-test.
[a] n Number of experiments (triplicates).
[b] NA – not applied.
[c] ND – not detected.

3.3. Biochemical analyses of wheat grains after ozone treatment

The carbonyl and carboxyl contents in the O_3-treated grain starch did not statistically differ from the control starch after 120 min of exposure (Table 2). After the 180 min exposure, however, there was a significant difference between the carboxyl contents of treated and control wheat starches.

Table 2. Carbonyl and carboxyl content of wheat starch isolates after 60 μmol/mol ozone treatment for 120 and 180 min.

Treatment	Carbonyl content (%)[a]	Carboxyl content (%)[a]
Control	0.198 ± 0.102	0.000
O_3 for 120 min	0.229 ± 0.049	0.037 ± 0.025
O_3 for 180 min	0.523 ± 0.210	0.079 ± 0.045*

*Symbols indicate statistically significant differences when compared with control group $p < 0.05$ by Tukey test.
[a] Results are the means of three determinations ± standard deviations.

The lipid peroxidation results showed no significant ($p > 0.05$) differences between the control and the O_3 treated samples (Fig. 2). Neither were any significant differences between treatments noted for the SDS-PAGE tests for reduced (Fig. 3a) and non-reduced (Fig. 3b) protein pattern changes in O_3 gas exposed wheat grains.

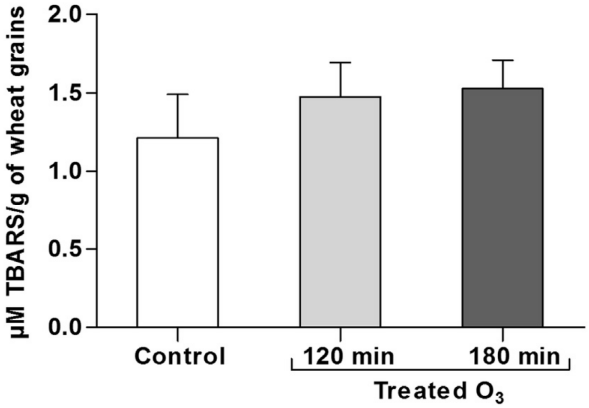

Fig. 2. Lipid oxidation of wheat grains after 60 µmol/mol ozone treatment for 120 min and 180 min (data are shown as mM TBARS/g of wheat grain). All treatments were not statistically significant when compared to the control group ($p > 0.05$) by Tukey test. however, there was a significant difference between the carboxyl contents of treated and control wheat starches.

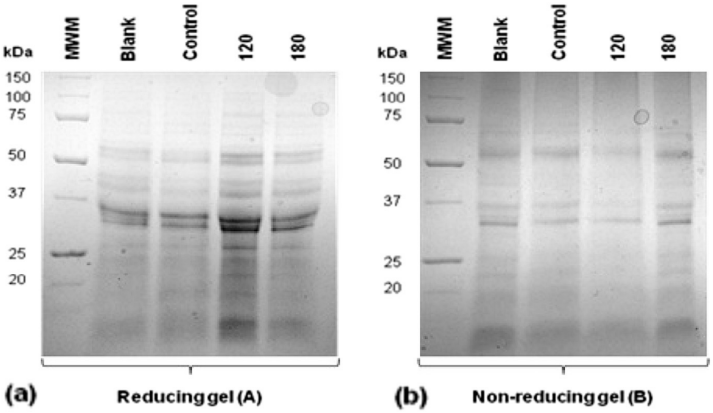

Fig. 3. SDS-PAGE analysis of total protein patterns in wheat grains in (a) the reduced patterns and (b) the non-reduced patterns. Lanes: M: molecular weight marker; B: blank test; C: Control; 60 mmol/mol ozone gas treatment for (1) 120 min and (2) 180 min.

3.4. XRD and SEM analyses of grain physical properties

No significant differences were noticed between control and treated groups in the XRD analysis (O_3 crystallinity), all groups showing the typical A-type X-ray diffraction pattern for cereal starches (Paris et al., 1999; Sandhu et al., 2012).

SEM analysis did not show any visible differences in grains morphology between O_3 treated and control samples. No damage to the microstructure characteristics of wheat grains was apparent after O_3 treatment. The external and internal surface of grain pericarp, brush pericarp and isolated starch remained intact (Fig. 4).

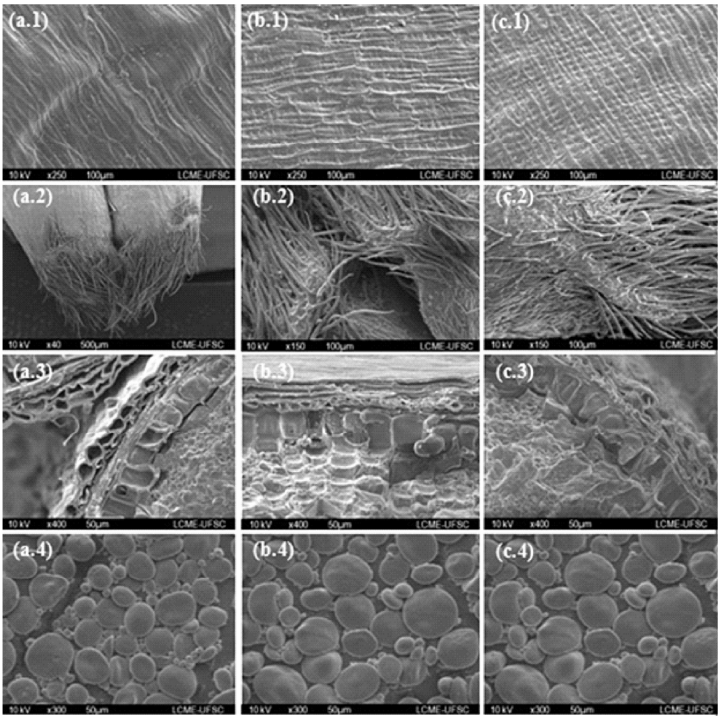

Fig. 4. SEM images of microstructure characteristics of wheat grains: (a) Control and treated with 60 µmol/mol ozone treatment for (b) 120 min and (c) 180 min of (1) external surface of the pericarp; (2) brush pericarp; (3) internal surface of the pericarp and (4) isolated starch.

3.5. Seed germination

After O_3 treatment at 60 µmol/mol, no effect on wheat germination was observed after 120 min of exposure, but the 180 min exposure reduced the germination capacity by 12.5% (Fig. 5). Additionally, no modifications to the length of the coleoptile or seminal root of germinated wheat seeds were observed after 60 µmol/mol O_3 treatment for 120 or 180 min (Fig. 6).

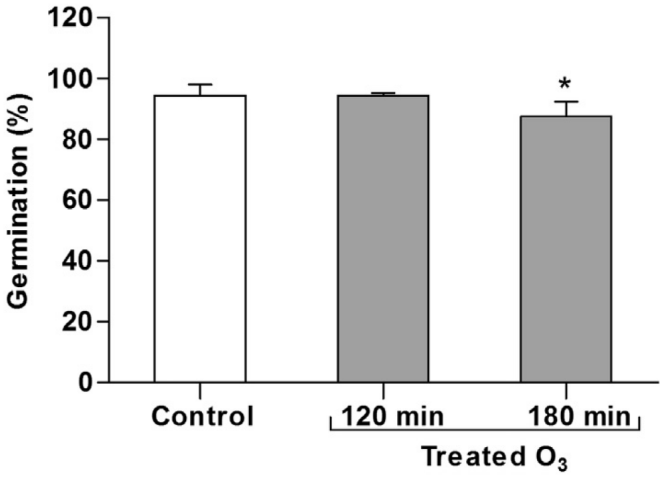

Fig. 5. Germination of wheat seeds after 60 µmol/mol ozone treatment for 120 min and 180 min. Symbols indicate statistically significant when compared with the control group ($p < 0.05$) by Tukey test.

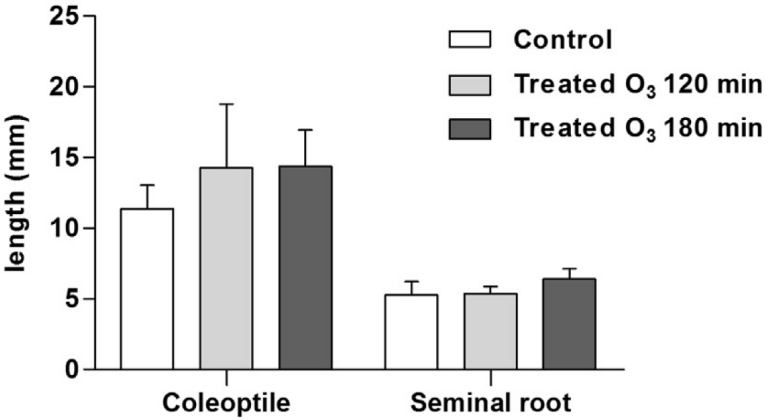

Fig. 6. Length (mm) of coleoptile and seminal root of wheat seeds after 60 µmol/mol ozone treatment for 120 min and 180 min. All treatments were not statistically significant when compared to the control group ($p > 0.05$) by Tukey test.

4. Discussion

4.1. Mycological analysis after ozone treatment

In recent decades, laboratory studies have shown that O_3 gas can be effectively used in order to control fungal growth in food such as barley, wheat, figs and Brazil nuts (Kottapalli et al., 2005; Wu et al., 2006; Zorlugenç et al., 2008; Scussel et al., 2011). For instance, with respect to wheat grains contaminated with *Fusarium*, Kottapalli et al. (2005) verified that O_3 treatment could reduce fungi growth by 24-36% after 15 min of O3 exposure at concentrations of 11 and 26 mg/g.

In the same context, in a previous in vitro study performed in our laboratory (Savi and Scussel, 2014), it was verified that after 60 min of O_3 exposure at 60 mmol/mol, the *F. graminearum* strain did not show growth until the eighth day of incubation. After 40 min of exposure, the growth was significantly smaller (38 mm) in comparison with the control (53 mm) at the end of the incubation. O_3 exposure also reduced conidia germination and caused morphological alterations of hyphae in this strain of *F. graminearum*.

Although it is recognized that *Fusarium* fungi do not persist after harvest of grain, the results presented in this paper are in accordance with the literature and show the high effectiveness of O_3 gas

treatment against *Fusarium* spp. in wheat grains, fungal viability being totally inhibited after exposure for 180 min at 60 µmol/mol.

4.2. DON analysis after ozone treatment

Ozone treatment can cause complete degradation or chemical changes in mycotoxins, decreasing the biological activity in terms of toxicity (McKenzie et al., 1998; Lemke et al., 1999). The DON degradation mechanism according to Criegee (1975) occurs when the ozone molecule undergoes 1-3 dipolar reaction with a double bond. This leads to the formation of ozonides (1,2,4-trioxolanes) from alkenes and ozone with aldehyde or ketone oxides as decisive intermediates, all of which have finite lifetimes (Cullen et al., 2009).

In the 1980s, Young (1986) looked into the effectiveness of O_3 in reducing DON levels in storage grains. When naturally contaminated whole wheat kernels (ca. 1 mg/g DON) were treated with dry O_3, there was no reduction in DON levels. In contrast, when vacuum oven dried ground corn artificially inoculated by F. graminearum (ca. 1000 µg/g DON) was treated with dry O_3, the half-life of DON disappearance was 2.5 h. This difference between the wheat and ground corn results may have been due to a matrix effect. The corn was ground and for this reason it became porous, making the treatment easier in relation to the whole wheat kernels where the O_3 may not have been able to penetrate. In contrast, in our study, the DON levels were strongly reduced to the limit of detection after O_3 treatment at a concentration of 60 µmol/mol for 180 min in grain pericarp and endosperm.

Young et al. (2006) also studied the degradation of trichothecenes with aqueous O_3, bubbling the gas through water for 30 min. DON total degradation was observed at an aqueous O_3 concentration of 25 ppm. However, the use of O_3 gas in storage is more feasible than aqueous O_3 due to the humidity in the grain bulk needing to be kept lowin order to avoid contamination by insects, fungi andmycotoxins.

It can thus be concluded that O_3 treatment is effective in reducing DON levels in contaminated wheat grains and haspotential in ensuring that DON levels can be kept below MTL 1000 µg/kg in stored whole wheat grains, in accordance with Brazilian legislation from 2016 (Brazil, 2011).

4.3. Physical and biochemical analysis of wheat grains after ozone treatment

Ozone reacts with the chemical constituents present in the grain's outer layer (seed coat) and, depending on the concentration and duration of O_3 exposure, can cause physical and biochemical changes in the grain layer. Other variables such temperature, moisture, grain characteristics and the presence of other organic matter such as insects and microorganisms on the grain surface may also affect grain quality.

Diffusion of O_3 into the grain depends on its physical characteristics. In this sense, it is important to bear in mind that the movement of O_3 inside a silo or column full of grain can happen in different directions (Tiwari et al., 2010). In addition, the presence of moisture also plays an important role in O_3 reactivity with grain because O_3 is soluble in water and this increases the contact be-tween gas and grain.

Mendez et al. (2003), investigated O_3 efficacy for the control of pests in stored wheat. They showed that O_3 treatment does not significantly change the bread-making properties of the hard wheat, including the tolerance of the dough to over mixing, absorption of water, dough weight, and proof height. In this study, the physical and biochemical characteristics in some grains after O_3 exposure were investigated at concentrations efficient for fungal growth inhibition and DON reduction in order to evaluate effects on grain quality and the results obtained were positive with no adverse changes evident.

O_3 is a powerful antimicrobial agent due to its potential oxidizing capacity (Khadre et al., 2001). After prolonged O_3 exposure, the gas may penetrate into grain starch causing its oxidation. Since the wheat grain has a high starch content (about 80%) oxidation of this grain portion is a possible side effect of O_3 exposure reducing the quality of the grain. According to Sánchez-Rivera et al. (2005), the oxidation grade in modified starches is determined by the concentration of carboxyl groups. The carboxyl content has a similar pattern to the carbonyl content in starches treated with O_3 treatment. In this study, the carboxyl content of wheat starch was only affected by O_3 treatment after 180 min. Differences in starch carboxyl content can also occur according to the botanical origin of the starch, type of oxidizing agent and reaction conditions, as discussed in Sangseethong et al. (2010).

The use of X-ray diffraction may also show if starch characteristics were modified due to O_3 treatment. Oxidation agents can cause relative crystallinity changes because the amylase and amylopectin chain can be damaged during the oxidation process (Kuakpetoon and Wang, 2006; Vanier et al., 2012). In this sense, it should be mentioned that our results were in accordance Sandhu et al. (2012), where the

wheat starch was treated with O_3 gas at a concentration of 1.5 µmol/mol and flow rate of 2.5 L/min for 30 min and 45 min. This did not produce a significant difference in starch crystallinity after O_3 treatment.

Lipid oxidation may occur when O_3 gas oxidizes the unsaturated lipids of the grain. Despite wheat grains having little lipid content (2.2%), it is important to evaluate the action of O_3 on the grain to check for oxidative rancidity. As found in the present work, a study with Brazil nuts (Scussel et al., 2011), which have high lipid content (60-70%), showed no change in the stability of lipids after an application of 10 µmol/mol O_3 for 90 min.

The protein modifications caused by oxidizing agents may reduce the flour quality of the processed products. According to Li et al. (2012), proteins under non-reducing conditions changed markedly due to treatment with 5 g/h O_3 over 30 and 60 min. Additionally, the intensity of the bands on the top of the separation gel increased significantly. Concerning the range of 31-45 kDa and 66.3-97.4 kDa, the reduction or disappearance of some bands was observed in the O_3-treated wheat flour proteins in comparison with the untreated control group. These changes indicated the potential for aggregation of low molecular weight proteins after O_3 treat-ment. In turn, in this study, a huge amount of protein aggregates were observed in both reduced and non-reduced SDS-PAGE pat-terns. But, this behavior has not been noticed when lower concentrations of O_3 gas were used.

It should be noted that some modifications (deformations and ruptures) may occur in the grains structure after exposure to the oxidizing agent. Thus, microscopy examination of the starch can be used to evaluate the quality of grains and starch gelatinization (Vanier et al., 2012). On the other hand, the biochemical changes are often associated with morphological changes. However, the O_3 treatment used in our work did not cause any physical alteration to the grain.

With respect to seed germination, the capacity of a grain seed to germinate determines whether it has field planting value. As a consequence, if O_3 affects the germination of wheat significantly, then it can only be used to preserve wheat stored for food purposes. Otherwise, O_3 could be used to preserve wheat all uses. According to Wu et al. (2006), by applying different O_3 doses at 0.016, 0.065, 0.16 and 0.33 mg (g wheat)$^{-1}$ min^{-1}, no effect on wheat germination was observed even after 60 min of ozonation. The wheat germination after an applied O_3 dose of 0.98 mg (g wheat)$^{-1}$ min^{-1} was barely affected after a 15-min exposure. However, after 20 and 30 min of ozonation, the germination rate reduced to 85.4% and 80.0%, respectively. In turn, after 45 min of

ozonation, the wheat germination was reduced to 61.3%.

5. Conclusion

O_3 gas treatment was effective in the inactivation of *F. graminearum* and reduction of DON contamination, especially in exposures of 60 µmol/mol for 120 min. These produced no alterations to the physical and biochemical characteristics of the whole wheat grains. Taking into account that the effectiveness of O_3 depends on several factors, including various environmental factors such as grain mass temperature, moisture and the surface characteristics, future investigations are needed for large-sized silo practical trials evaluating efficacy of O_3 treatments against a range of fungi on greater amounts of stored wheat.

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11 CAPÍTULO 9

Eficiência do tratamento com ozônio gasoso na inibição do crescimento de *Aspergillus & Penicillium* e degradação de micotoxinas de armazenamento nos grãos de trigo (*Triticum aestivum* L.)

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Ozone Treatment Efficiency in *Aspergillus* and *Penicillium* Growth Inhibition and Mycotoxin Degradation of Stored Wheat Grains (*Triticum aestivum* L.)

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ABSTRACT

The efficacy of ozone (O_3) gas treatment to inhibit the strains growth of *Aspergillus flavus* and *Penicillium citrinum* and of aflatoxin and citrinin (CTR) to degrade in wheat grains were evaluated. The tests were performed in laboratory pilot silos containing grains artificially contaminated by these fungi or mycotoxins. Briefly, the samples were divided into control groups (no O_3 gas) and O_3-treated groups (40 and 60 μmol/mol), which were exposed for 30, 60, 120 and 180 min. In the highest concentration of O_3 exposure (60 μmol/mol), *A. flavus* and *P. citrinum* showed complete growth inhibition after 180 min. In addition, regarding the same O_3 exposure, the AFB_1 and AFB_2 levels were significantly reduced (12.51 and 41.06 μg/kg) when compared with control (231.88 and 265.79 μg/kg). Moreover, the CTR levels were significantly decreased after O_3 treatment in both concentrations (40 and 60 μmol/mol) after 180 min of O_3 exposure (103.64 and 42.90 μg/kg) when compared with control group (146.85 and 173.51 μg/kg).

Keywords: fungi, mycotoxins, whole wheat grains, ozone gas.

PRATICAL APPLICATIONS

The *Aspergillus flavus* and *Penicillium citrinum* strains have been found in stored wheat grains. These fungi can be responsible for aflatoxins and citrinin formation under specific temperature and humidity storage conditions. In order to overcome this problem, ozone has been studied in laboratory scale to control fungal growth. It is possible to say that this is an attractive practice and

is effective for the food industry because it decomposes to molecular oxygen without leaving residues, and furthermore is considered safe by the U.S. Food and Drug Administration.

INTRODUCTION

The *Aspergillus flavus* and *Penicillium citrinum* strains have been usually found in storage grains, as discussed in several studies (Berghofer *et al*. 2003; Riba *et al*. 2008, 2010; Roigé *et al*. 2009). *A. flavus* as well as *A. parasiticus* and *A. nomius* (Hussein and Brasel 2001) are aflatoxins (AFLs) producers frequently observed in tropical and subtropical climate areas. AFB_1, AFB_2, AFG_1 and AFG_2 are among the most known aflatoxins in the world. For example, AFB1 is the predominant and most potentially mutagenic, teratogenic and hepatocarcinogenic according to the International Agency for Research on Cancer (IARC 1993). In turn, *P. citrinum* is the main citrinin (CTR) producer, which is also produced by other filamentous fungal species of *Aspergillus*, *Penicillium* and *Monascus* (Cole 1986; Blanc *et al*. 1995). In summary, the most important toxic effect of this mycotoxin can be associated with nephrotoxicity. In addition, it is also known for its cytotoxic, genotoxic, mutagenic, immunotoxic and teratogenic properties (Kogika *et al*. 1993; Kumar *et al*. 2007). For this reason, CTR has been considered once as a potential human risk factor because of Balkan endemic nephropathy disease, originally described as a chronic tubulointerstitial kidney disease in southeastern Europe (Pfohl-Leskowicz *et al*. 2002; Bamias and Boletis 2008).

The presence of both aforementioned mycotoxins can be found in a wide variety of important agricultural commodities (Vrabcheva *et al*. 2000; Kononenko and Burkin 2008; Muthomi *et al*. 2008; Li *et al*. 2012; Soleimany *et al*. 2012; Zaied *et al*. 2012). Nevertheless, they are often found in wheat grains (Comerio *et al*. 1998; Giray *et al*. 2007; Kononenko and Burkin 2008; Muthomi *et al*. 2008; Riba *et al*. 2010; Lutfullah and Hussain 2012; Soleimany *et al*. 2012; Zaied *et al*. 2012), which is the focus of this study.

In the last 2 years in Brazil, around 5.09 million tons of wheat was harvested per year, mainly in southern Brazil (94%). However, the wheat grain annual national demand is approximately equal to 10 million tons. Consequently, Brazil

needs to import annually more than 5 million tons of wheat grain, especially from Argentina (Conab 2012). The exposure of grains and seeds in these subtropical climate regions may cause their deterioration and contamination by fungi and mycotoxins during storage periods, due to environmental factors such as temperature and humidity.

In order to mitigate problems related to grains storage, the Brazilian Federal Regulation has set in 2012 the maximum levels (ML) for AFLs in cereals and products derived from cereals at 5 μg/kg (Brasil 2011), similar to the levels defined in Europe by the (European Union Commission 2006) (4 μg/kg). However, there is no specific legislation for CTR because it is difficult to establish widely acceptable limits for this mycotoxin due to a lack of suitable analytical routine methods or its unstable behavior. In any case, the chemical food decontamination strategies have gradually been used to reduce fungal and mycotoxins growth in grains, especially in laboratory scale. In this sense, it is possible to highlight ozone (O_3) gas use. It is a powerful antimicrobial agent owing to its potential oxidizing capacity (Khadre *et al*. 2001) and, additionally, decomposes the molecular oxygen without leaving residues.

For instance, the U.S. Food and Drug Administration classified O_3 as generally recognized as safe (GRAS) (FDA-United States Food and Drug Administration 1982) to be used in bottled water and in food processing (Graham 1997). Moreover, the Food and Agriculture Organization (FAO-Food and Agricultural Organization of the United Nations 1996) recognizes the potent disinfectant characteristics of O3.

The O_3 gas has been effectively used to control fungal growth in laboratory scale (Kottapalli *et al*. 2005; Wu *et al*. 2006; Zorlugenç *et al*. 2008; Scussel *et al*. 2011; Savi *et al*. 2014b) and consequently to decrease mycotoxin contamination in peanuts, figs, Brazil nuts, wheat and artificially contaminated corn (Dwarakanath *et al*. 1968; Zorlugenç *et al*. 2008; McDonough *et al*. 2011; Scussel *et al*. 2011; Savi *et al*. 2014b).

Therefore, this study aims to evaluate the effectiveness of O_3 gas treatment against *A. flavus* and *P. citrinum* strains growth as well as AFLs and CTR degradation in wheat grains.

MATERIALS AND METHODS

Samples

About 50 kg of wheat grain was taken from vertical silos in 2012 after storage for 3 months. Samples were sent to the Laboratory of Mycology, Mycotoxicology and Food Contaminants of the Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil after cleaning and drying (up to a maximum of 60C) steps in the storage unit under responsibility of the Brazilian Agricultural Research Corporation (EMBRAPA Wheat), packed in a polyethylene bag and stored once again at 4 ºC for analysis in this study.

Fungi Strains

The fungi strains *A. flavus* and *P. citrinum* were obtained from the Laboratory of Mycology, Mycotoxicology and Food Contaminants culture collection at the Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil.

Instruments

Initially, some materials/instruments used in this study were: O3 gas generator (5–60 μmol/mol), corona discharge, Interzone (Jundiai, São Paulo, Brazil); impurities remover (Alvorada, Rio Grande do Sul, Brazil); flow meter, Protec (São Paulo, Brazil). For mycological tests, the equipment required were: light microscopes, CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); autoclave, Phoenix (Araraquara, São Paulo, Brazil); microwave oven, Philco (São Paulo, Brazil); laminar flow cabinet, Veco (Campinas, São Paulo, Brazil); fume cabinet, Quimis (Diadema, São Paulo, Brazil); rotary shaker, Marconi (Piracicaba, São Paulo, Brazil) and microbiological incubator, Quimis.

To get the moisture content (mc), a drying oven Olidef-cz (Ribeirao Preto, São Paulo, Brazil) was used.

High-performance liquid chromatography (HPLC) system equipped with an isocratic pump model 805 and fluorescence detector (FLD, excitation 335 nm and emission 440 nm) all from Gilson (Middleton, WI), were used to perform mycotoxin analysis. In addition, was used the C18 columns 5 μm particle size with 250 × 4.60 mm (Phenomenex-Madrid Avenue,

Torrance, CA) and 150 × 4.60 mm (ACE-Scotland, UK), for AFLs and CTR analysis, respectively.

Culture Media and Chemicals

Culture Media. Potato dextrose agar (PDA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil).

Chemicals Used. AFLs and CTR standard from Sigma Aldrich Chemicals (St. Louis, MO); acetonitrile and methanol with liquid chromatography (LC) grade, chloramphenicol, hydrochloric acid, potassium phosphate monobasic, sodium phosphate, sodium chloride, potassium chloride, potassium bromide, potassium iodide solution, sodium thiosulfate, nitric acid and sulfuric acid were obtained from Vetec (Duque de Caxias, Rio de Janeiro, Brazil). The water was obtained from a Milli-Q system 18.2 MΩ/cm.

Other Materials. The immunoaffinity columns from Citri-Test HPLC Vicam (Milford, MA) and NeoColumn Aflatoxin DR (direct read), AOAC RI 081002 (2010), Neogen Corporation (Lansing, MI) were used to perform mycotoxin analysis.

Samples Strain Inoculation and Toxin

Spiking Prior to Ozone Treatment

For mycological analysis, a solution (5 mL) of Tween 80 containing 1×10^4 spores/mL of *A. flavus* and *P. citrinum* was sprayed on wheat grains (25 g) to artificially contaminate the samples. In turn, wheat grains (12.5 and 10 g) were spiked using 100 μL of aflatoxins mix (AFLs) and CTR standards solutions (2.5 and 100 μg/mL) to propitiate the mycotoxin analysis. In order to know the mc (2 g) after artificial contamination, the wheat grains were submitted to drying in an oven (105 ºC) up to constant weight by means of gravimetric method, according to Association of Official Analytical Chemists AOAC (2005). After the steps of contamination with fungi and mycotoxins, the grains had mc equal to 16 and 14%.

Ozone Gas Wheat Treatment in the Storage

Laboratory Pilot Silos

The laboratory pilot silos with capacity of 25 × 10 cm (length × diameter) were made with vinyl polychloride tubes containing only two apertures: one to put O_3 gas (bottom) and another to remove it (top). Thus, they were filled with 350 g of wheat grains and, at the top (above grains), a polyamide screen surface was placed in order to support the artificially contaminated grains, on which would be performed the mycological (25 g) and mycotoxin (12.5 and 10 g for AFLs and CTR) analysis after O_3 gas application. Afterwards, they were divided into control group (no O3 gas) and treated group (40 and 60 μmol/mol), which were exposed for 30, 60, 120 and 180 min in a room at 25 ºC ± 0.5 ºC. The O_3 gas generator system followed the procedures detailed by Giordano *et al*. (2012) with minor modifications. In summary, the compressed air pump was connected to an air impurities remover to get rid of solid particles and humidity. After that, the filtered air was driven to the adjusted flow meter for 1 L/min and then the O_3 generator was calibrated to reach a concentration of 40 or 60 μmol/mol. The O_3 production by the generator (5–60 μmol/mol) used the corona discharge process, which was produced by electrical discharge caused by the passage of air or pure oxygen (O_2) between the two electrodes, generating the conversion of O_2 to O_3. The O_3 gas produced was injected through a tube into the input aperture of each chamber. The control chamber (without O_3 gas) was ventilated with "room air" with the same flow (1 L/min). The O_3 gas concentration was measured by the iodine metric titration test from the output of the O_3 generator. The gas was bubbled into a potassium iodide solution (50 mL), acidified with 2.5 mL of sulfuric acid 1 N (pH below 2.0). The solution was titrated with sodium thiosulfate 0.005 N using a starch solution as indicator, according to American Public Health Association (APHA 1999).

Mycological Analysis after Ozone Treatment

The enumeration technique was applied to evaluate the total fungi load (Silva *et al*. 2010). Twenty-five grams of each artificially contaminated wheat sample was added to 225 mL of 0.1%

peptone dissolved in water under sterile conditions. The mixture was stirred in a rotary shaker for 2 min and afterwards the dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were performed. Aliquots of 0.1 mL of each dilution were spread (in duplicate) on the surface of the PDA medium containing chloramphenicol 100 mg/L and incubated for 7 days at 28 ºC in the dark. The results were expressed in colony forming units per gram (cfu/g).

Mycotoxins Analysis after Ozone Treatment

Aflatoxins. Whole wheat grains samples were analyzed using immunoaffinity columns for clean-up step and LC/FLD for detection, according to Neogen protocol AFLs DR HPLC (Neogen, 2013), with some modifications. Briefly, 12.5 g of each artificially contaminated wheat sample were ground in an industrial blender jar with 2.5 g of NaCl and 62.5 mL of LC-grade methanol 70%. The mixture was blended for 2 min, followed by filtration. The filtrate (7.5 mL) was diluted in 15 mL of 10 mM phosphate buffered saline (PBS) and then filtered off. Next, 7.5 mL of this filtrate was cleaned by immunoaffinity column in a flow rate of one drop per second. After washing the column with 10 mL of LC-grade water, the toxin was slowly eluted with 0.5 mL of 100% LC-grade methanol and 0.5 mL of LC-grade water. The eluate was evaporated to dryness using heating block set at 40 ºC with gentle nitrogen stream and the dry residue was then redissolved with 100 μL of mobile phase (water: methanol: acetonitrile [60:20:20 v/v/v] added 119 mg potassium bromide and 47.6 μL nitric acid). The extract (20 μL) was injected into the LC/FLD system. The mobile phase was delivered in a flow constant rate of 1 mL/min. AFLs levels quantification was performed by measurement of peak area at AFLs retention time compared with the standard solutions used for calibration curve (0.035–4 μg/mL for AFB_1; 0.00065–2 μg/mL for AFB_2; 0.035–4 μg/mL for AFG_1; 0.00091–2 μg/mL for AFG_2, with a correlation factor equal to 0.990, 0.993, 0.991 and 0.993, respectively). Recovery was determined by spiking AFLs-free samples of wheat with AFLs concentrations of 8 and 12 μg/kg on the same day and at the same HPLC conditions.

Citrinin. Whole wheat grains samples were analyzed using immunoaffinity columns for clean-up step and LC/FLD for

detection, according to Vicam protocol Citri-test HPLC (Vicam, 2013), with some modifications. In summary, 10 g of each artificially contaminated wheat sample were ground in an industrial blender jar with 25 mL of LC-grade methanol:water (70:30, v/v). The mixture was blended during 1 min and filtered off. The filtrate (1 mL) was diluted in 24 mL of 10 mM phosphoric acid (pH 7.5). Next, it was filtered off and 10 mL of this filtrate was cleaned by immunoaffinity column in a flow rate of one drop per second. After washing the column with 2.5 mL of LC-grade phosphoric acid (pH 7.5), the toxin was slowly eluted with 1 mL of LC-grade methanol:10 mM phosphoric acid (70:30, v/v). The eluate was collected and injected into the LC/FLD system. The mobile phase was 10 mM phosphoric acid pH 2.5:acetonitrile (30:70, v/v) delivered in a flow constant rate of 0.5 mL/min. CTR levels quantification was performed by measurement of peak area at CTR retention time compared with the standard solutions used for calibration curve (0.01, 0.1, 1, 10 and 100 μg/mL) with a correlation factor equal to 0.998. Recovery was determined by spiking CTR-free samples of wheat with CTR concentrations of 20 and 200 μg/kg on the same day and at the same HPLC conditions.

Statistical Analysis

The obtained data were analyzed using analysis of variance (ANOVA) and Bonferroni's post-test or Dunnett's multiple comparison test. The results were presented as mean ± standard deviation and the values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Ozone Treatment on *A. flavus* and *P. citrinum* Growth

Firstly, it is important to highlight that *A. flavus* growth was significantly reduced (8.5×10^1 and 5.35×10^1 cfu/g) after 30 min of O_3 exposure in concentrations of 40 and 60 μmol/mol when compared with the control group (44×10^1 cfu/g), which represents 80.7 and 87.8% spores inhibition. However, only O_3 exposure at 60 μmol/mol totally inhibited *A. flavus* growth after

180 min (Fig. 1a). In addition, *P. citrinum* also was significantly reduced (8.4×10^1 and 6.9×10^1 cfu/g) after 30 min of O_3 exposure in concentrations of 40 and 60 µmol/mol, when compared with the control group (25.9×10^1 cfu/g), which represents 67.6 and 73.4% spores inhibition. The total inhibition of *P. citrinum* growth occurred after O_3 treatment with 60 µmol/mol during 180 min of exposure (Fig. 1b).

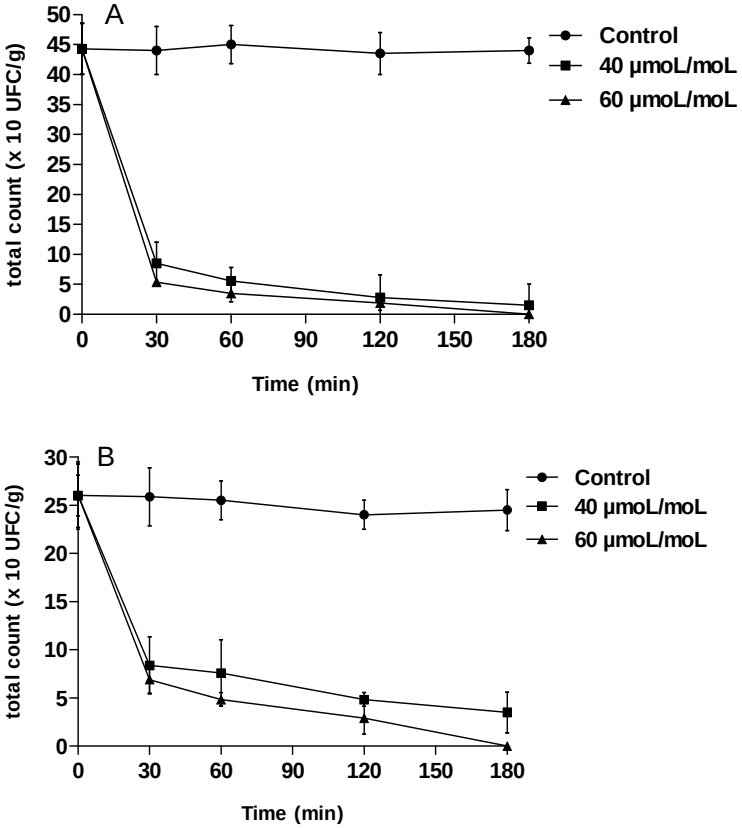

Figure 1. Ozone Gas Effect on (A) *A. flavus* and (B) *P. citrinum* growth at Different Concentrations and Exposure Time (40 and 60 µmol/Mol; 30 to 180 min). All treatments were statistically significant when compared to Control Group ($P<0.05$) by Bonferroni Post-Test.

With respect to the *in vitro* study performed previously in our laboratory (Savi and Scussel 2014a), *P. citrinum* was also not able to grow in culture media PDA after passing through 120 min of O_3 gas exposure (60 μmol/mol).

Moreover, its growth was significantly smaller (19, 16 and 10 mm) than the control group (23 mm) in differents times of O_3 gas exposure (40, 60 and 90 min). On the other hand, *A. flavus* presented a different behavior. Regarding distinct exposure time, it was more resistant to the treatment and even then had grown in culture media. Nevertheless, the growth was significantly smaller after 120 min of O_3 gas exposure (60 mm) when compared with the control group (78 mm). This *in vitro* study showed that O_3 exposure reduced the conidia germination and caused hyphae morphological alterations of the fungi.

Although other studies have shown the efficiency of O_3 treatment in combating *Aspergillus* genera, there is no published study that presents the O_3 exposure effects on *P. citrinum* toxigenic species in grains according to the author's knowledge. In order to justify the previous statement, Kells *et al*. (2001) observed O_3 efficacy against *A. parasiticus* in storage corn grains, for instance. They showed that the number of viable *A. parasiticus* conidia on the grain surface was reduced around 63% when grain was exposed to 50 μmol/mol for 3 days. The same idea could be noticed in Brazil nuts (Giordano *et al*. 2012). The O_3 treatment in a concentration of 31 μmol/mol during 5 h of exposure was able to successfully reduce completely *A. flavus* and *A. parasiticus* (initial: 6.7×10^4 cfu/g), since day one after application.

Effect of Ozone Treatment on Aflatoxin and Citrinin Degradation

The LC/FLD method for AFLs and CTR chromatographic separation and the validation parameters (linearity, limit of detection [LOD], limit of quantification [LOQ], reproducibility, repeatability and recovery) showed to be quite adequate. Under the chromatographic conditions applied, the AFLs retention time (Rt) were 19 ± 0.5, 15 ± 0.5, 14 ± 0.5 and 11 ± 0.5 min for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Linearity was confirmed using the calibration curve for each AFLs concentration, which were linear at 0.035-4 μg/mL/ 0.00065-2

μg/mL/ 0.035-4 μg/mL and 0.00091-2 μg/mL for AFB_1/ AFB_2/ AFG_1 and AFG_2, with a correlation factor equal to 0.990/ 0.993/ 0.991/ 0.993, respectively. The LOD/LOQ (signal-to-noise ratio = 3/10) were 0.26/3.1, 0.002/0.02, 0.28/1.41 and 0.005/0.03 μg/Kg for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. The mean recovery of the extraction method considering the concentrations equal to 8 and 12 μg/kg was 87 and 90% for AFB_1, 90 and 92% for AFB_2; 85 and 87% for AFG_1; 98 and 97% for AFG_2. On the other hand, the Rt was 20 ± 0.5 min for CTR, considering a linear calibration curve in concentrations of 0.01; 0.1; 1; 10 and 100 μg/mL, with a correlation factor equal to 0.998. The LOD and LOQ were 0.2 and 1.2 μg/kg. The mean recovery of the extraction method concerning the concentrations of 20 and 200 μg/kg was around 72.86 and 90.90%. The O3 treatment may degrade or cause chemical changes in mycotoxin and then reduce its biological activity in terms of toxicity (McKenzie *et al*. 1998; Lemke *et al*. 1999). After 60 μmol/mol O_3 treatment, the AFLs levels were significantly reduced to 12.51, 41.06, 47.96 and 37.81 μg/kg (control group - 231.88, 265.79, 239.92 and 199.44 μg/kg) after 180 min, which represent 94.6, 84.5, 80.0 and 81.0% of reduction for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. On the other hand, after 40 μmol/mol O3 treatment in the same exposure time, only AFB_1 and AFB_2 were significantly reduced to 43.78 and 68.79 μg/kg (88.6 and 74.8% of reduction). Among the AFLs, it is possible to say that AFB_1 and AFB_2 were the mycotoxins that presented the best results in relation to treatment in the two concentrations (40 and 60 μmol/mol), once they were significantly reduced after 30 min of O_3 exposure (Table 1).

The CTR was decreased after O_3 treatment in both concentrations (40 and 60 μmol/mol) after 180 min of exposure to 103.64 and 42.90 μg/kg (control group - 146.85 and 173.51 μg/kg), which represents 29.4 and 75.3% of reduction. The treatment in the concentration of 60 μmol/mol showed the greatest results, once it substantially reduced after 30 min of exposure (123.21 μg/kg - 29% of reduction) when compared with control group (173.51 μg/kg) (Table 2).

Efficiency results of O_3 treatment against fungi and mycotoxins have been reported in the scientific literature, depending on the concentrations used, as well as their application forms. Previous studies showed that O_3 was effective in AFLs degradation in several foods, especially nuts and fruits, such as

pistachios, peanuts, red pepper and dried figs (Akbas and Ozdemir 2006; Inan *et al*. 2007; Zorlugenç *et al*. 2008; Alencar *et al*. 2012). Nevertheless, there are few studies with respect to O_3 gas treatment on AFLs degradation in cereals, such as paddy rice, wheat and corn (Wang *et al*. 2010; McDonough *et al*. 2011; El-Desouky *et al*. 2012; Luo *et al*. 2014), and, more important, the effect of O_3 on CTR degradation in grains has not been assessed yet in literature, as to the knowledge of the authors. For example, in the study proposed by Luo *et al*. (2014), authors only verified that AFB_1 was easily degraded by O_3 in corn. When corn with 13.47% mc was exposed to O_3 at the concentrations of 40, 65 and 90 μmol/mol for 40 min, the AFB_1 degradation rates were 41.1, 56.2 and 88.1%, respectively. In wheat grains contaminated artificially with *A. flavus* (105 spores/mL), they did not observe any amount of AFB1 in the wheat samples after O_3 treatment (20 and 40 μmol/mol) for 10, 15 and 20 min (El-Desouky *et al*. 2012). In field trials for artificially contaminated corn, the effect of O3 treatment on AFLs-contaminated corn revealed that total AFLs was reduced to 5.7 and 3.1 mg/kg of corn when compared with 17.6 and 15.7 mg/kg of non-O_3 (air) treated, which indicated a reduction of AFLs by 20-30% due to O_3 treatment (McDonough *et al*. 2011). In another study, which analyzed the mechanism of AFB1 degradation (McKenzie *et al*. 1997), O_3 firstly reacts across the C8 C9 double bond of the furan ring of AFB1 through electrophilic attack based on the Criegee mechanism, and then produces intermediate products. The destruction of the C8 C9 double bond on the furan ring means that the toxicity of AFB_1 has been reduced or even disappeared. Therefore, our study showed the efficiency of O_3 treatment in the preservation of storage wheat grains against *A. flavus* and *P. citrinum*, as well as AFLs and CTR often found in this food. Moreover, the application of O_3 gas in concentrations and exposure times used in this study (40 and 60 μmol/mol) have not affected several quality attributes in the wheat grains, such as isolated starch oxidation and X-ray diffraction, lipid peroxidation, total protein profile and seed germination (Savi *et al*. 2014b). In this context, O_3 gas has a potential usefulness in the grains storage treatment to reduce the mycotoxins levels below of Brazilian regulation and to improve the quality of storage wheat grains.

CONCLUSION

The O_3 gas treatment was effective in inactivation of *A. flavus* and *P. citrinum* growth, as well as AFLs and CTR degradation in wheat grains, especially under 180 min at 60 μmol/mol concentration. Considering AFB_1, more toxic among the AFLs, it is important to highlight that this was highly reduced by O_3 treatment. Finally, according to the author's knowledge, this is the first work that has assessed the effect of O_3 on CTR degradation in grains. Nevertheless, future investigations are needed in order to verify the effectiveness of O_3 in large size silos industry, where there is a wide scale of grains stored.

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Table 1. Ozone gas effect on aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2) degradation of two concentrations and different times of exposure

O_3 treatment				**Aflatoxins**							
Group		**Concentration (µmol/mol)**	**Exposure (min)**	**AFB_1**	**Reduction**	**AFB_2**	**Reduction**	**AFG_1**	**Reduction**	**AFG_2**	**Reduction**
				mean ± µg/kg	**%**	**mean ± µg/kg**	**%**	**mean ± µg/kg**	**%**	**mean ± µg/kg**	**%**
Treated	I	40	30	204.80 ± 44.40*	46.8	69.99 ± 40.16*	74.3	101.82 ± 73.55	NA	89.26 ± 34.83	NA
			60	69.97 ± 5.9*	81.8	65.63 ± 64.03*	75.9	102.41 ± 84.83	NA	88.55 ± 43.57	NA
			120	27.25 ± 28.26*	92.9	63.54 ± 42.60*	76.7	88.46 ± 7.72	NA	85.59 ± 32.18	NA
			180	43.78 ± 11.30*	88.6	68.79 ± 89.49*	74.8	87.16 ± 48.94	NA	84.90 ± 22.23	NA
Control		No O_3	NA	385.32 ± 73.38	NA	272.52 ± 85.70	NA	203.89 ± 131.12	NA	146.55 ± 26.17	NA
Treated	II	60	30	109.56 ± 4.5*	52.7	59.76 ± 35.00 *	77.5	154.28 ± 20.10	NA	160.77 ± 44.12	NA
			60	24.75 ± 19.86*	89.3	98.82 ± 48.34*	62.8	86.47 ± 24.87*	63.9	157.21 ± 65.00	NA
			120	24.27 ± 46.60*	89.5	50.34 ± 73.75*	81.0	54.19 ± 87.66*	77.4	135.54 ± 11.74	NA
			180	12.51 ± 5.0*	94.6	41.06 ± 13.98*	84.5	47.96 ± 7.41*	80.0	37.81 ± 4.34*	81.0
Control		No O_3	NA	231.88 ± 27.78	NA	265.79 ± 14.88	NA	239.92 ± 50.05	NA	199.44 ± 92.63	NA

NA, no applicable

* Symbols indicate statistically significant when compared with Control Group $p<0.05$ by Dunnett´s Multiple Comparison Test

Table 2. Ozone gas effect on citrinin (CTR) degradation of two concentrations and different times of exposure

Group		O_3 Treatment		Citrinin	
		Concentration (µmol/mol)	**Exposition time (min)**	**CTR (mean ± µg/kg)**	**Reduction %**
Treated	I	40	30	146.77 ± 6.43	NA
			60	144.43 ± 28.14	NA
			120	129.23 ± 0.11	NA
			180	103.64 ± 20.92 *	29.4
Control		No O_3	NA	146.85 ± 12.62	NA
Treated	II	60	30	123.21 ± 0.078 *	29.0
			60	94.65 ± 13.63 *	45.4
			120	93.94 ± 7.73 *	45.8
			180	42.90 ± 10.22 *	75.3
Control		No O_3	NA	173.51 ± 14.72	NA

NA, no applicable

* Symbols indicate statistically significant when compared with Control Group $p<0.05$ by Dunnett´s Multiple Comparison Test

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12 CAPÍTULO 10

Remoção de resíduos de agrotóxicos em grãos de trigo (*Triticum aestivum* L.) armazenados por gás ozônio

ARTIGO SUBMETIDO: Savi, Geovana Dagostim, Scussel, V.M. Removal of pesticides residual in stored wheat grains (*Triticum aestivum* L.) by ozone gas.

Removal of pesticides residual in stored wheat grains (*Triticum aestivum* L.) by ozone gas

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Abstract

The effectiveness of ozone (O_3) gas treatment on the removal of residual organophosphate (fenitrothion) and pyrethroid (deltamethrin) pesticides in wheat grains (*Triticum aestivum* L.) by O_3 gas were evaluated. The fenitrothion content was decreased after O_3 (60 µmol/mol) treatment after 180 min of exposure (69.4% of reduction). In addition, the treatment was effective when the grains presented the highest humidity parameters (moisture content: mc - 20% and water activity: a_w - 0.9). The same occurs with deltamethrin, which was significantly reduced (67.5, 88.0 and 89.8% for 60, 120 and 180 min of O_3 exposure, respectively). On the other hand, wheat grains that present the lower mc and a_w (12% and 0.6) had 80.5% and 85.7% of reduction after O_3 exposure of 120 and 180 min, respectively. The possibility of O_3 application in storage unit may be studied as effective method for chemicals contaminants degrade, as pesticides residues, commonly used on the storage wheat grains.

Keywords: ozone gas; pesticides; wheat grains; storage.

1. Introduction

Pesticides treatments, such as with organophosphate and pyrethroid insecticides, are carried out in order to avoid wheat grain losses during storage, mainly due to frequent attack of insects. They can have many benefits, such as an increase profitability of production and cereal quality. On the other hand, they have toxic effects to the environmental and to living organisms when applied improperly, which leads to

contamination of water, air and soil (Warner et al., 2005; Fernandez-Alvarez et al., 2008; Lozowicka et al., 2014).

Insecticides are widely used in the storage units. They are sprayed by liquid *via* on wheat grains when conveyor carriers before of the grain storage step in the silos to prevent insect infestation. Fenitrothion and deltamethrin pesticides are example of insectides often used on wheat grains (Fig. 1).

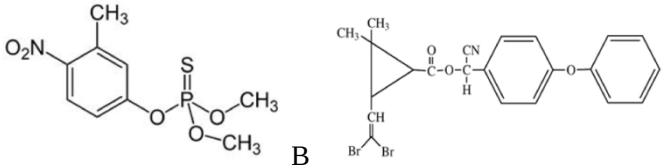

Fig. 1. Chemical structure of (A) Fenitrothion and (B) Deltamethrin.

The deltamethrin is a pesticide classified as a pyrethroid, which are synthetic inseticides having chemical structures similar to the natural chemicals, pyrethrins, produced by the flowers of pyrethrums, the old world plants of the genus *Chrysanthenum*. Deltamethrin intoxication may cause serious health effects to human such as paraesthesia headache, dizziness, nausea and skin irritation (FAO, 1999). It is applied on stored wheat grain for the control of: *Laemophloeus minutus* (beetle); *Tribolium castaneum* (brown beetle); *Sitophilus oryzae* (weevils); *Rhizopertha dominica* (grain weevils) and *Sitotroga cerealella* (moth) (Agrolinkfito, 2012). On the other hand, the fenitrothion is classified as an organophosphate insecticide, which may cause severe environmental and health hazards to organisms, including humans (FAO, 1999). Fenitrothion is used for the control of *Sitophilus oryzae* (weevils) infestation (Agrolinkfito, 2012).

Despite of their efficiency to avoid insect infestation, these insecticides have restrictions due to persistent problems in the grains and by-products as residues besides the occurrence of insect resistance (Embrapa, 2013). Therefore, to ensure the food safety, the control of pesticide residues is based on the maximum residue limits (MRLs) and storage security intervals. For wheat grains, the MRL of Brazilian regulation is 1.0 mg/kg for fenitrothion and deltamethrin. The storage intervals periods are about of 30 and 120 daysto deltamethrin and fenitrothion, respectively. In addition, the white bread has MRL (0.2 mg/kg)

only for fenitrothion (Brazil, 2009). According to EU (2005), the MRL are about 0.5 and 2.0 mg/kg for both pesticides (fenitrothion and deltamethrin, respectively). On the other hand, the Codex Alimentarius propose the MRL of 2.0 and 0.3 mg/kg for fenitrothion and deltamethrin in white flour and 5.0 mg/kg for fenitrothion in integral flour. Finally, the MRL for bran is of 200 and 5.0 mg/kg for fenitrothion and deltamethrin (Codex, 2005).

Agriculture crops may not be marketed when they contain pesticides exceeding the residual limit (Yamaguchi, 2006). In addition, food pesticide residues have toxic effects to human and animal health. Therefore, the development of new methodologies to eliminate residual pesticides has been aimed for several studies (Ikehata and Gamal El-din, 2005a, 2005b, 2006; Gabler et al., 2010; Ikeura et al., 2011; Kusvuran et al., 2012; Ikeura et al., 2013). In this sense, an emerging strategy is regarded to ozone (O_3) gas treatment. O_3 is a powerful antimicrobial agent owing a potent oxidizing capacity (Khadre et al., 2001) and, additionally, undergoes self-decomposition to molecular oxygen without leaving food residues. For instance, the US Food and Drug Administration (FDA) classified O_3 as *Generally Recognized as Safe* (GRAS) (FDA, 1982) to be used in bottled water and in food processing (Graham, 1997). Moreover, the Food and Agriculture Organization (FAO, 1994) recognize the potent disinfectant characteristics of O_3.

The O_3 application in the wheat grains may be promising for the quality and food safety, in previous studies since demonstrated efficacy in reducing chemicals contamination, such as mycotoxin in grains (McDonough et al., 2011; Scussel et al., 2011; Savi et al., 2014) and also removal of pesticide residues in food, especially vegetables and fruits (Gabler et al., 2010; Ikeura et al., 2011; Kusvuran et al., 2012; Ikeura et al., 2013).

O_3 treatment aiming mycotoxin degradation in stored grains could be doubly advantageous, since it can reduce other chemical contaminants, such as pesticides residues. Therefore, this study aim to evaluate the effectiveness of O_3 gas treatment in the removal of residual organophosphate (fenitrothion) and pyrethroid (deltamethrin) insecticides commonly used in wheat grains (*Triticum aestivum* L.).

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Fenitrothion and deltamethrin analytical standards were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA); acetonitrile and methanol with LC grade, dichloromethane and phosphoric acid were purchased from Vetec (Duque de Caxias, RJ, Brazil). The Milli-Q water was obtained from Synergy (Millipore, USA) with resistivity of 18.2 MΩ/cm. *Other materials:* the solid phase extraction (SPE) columns Strata-X (200 mg/6mL), Phenomenex (Madrid, Avenue, Torrance, USA).

2.1.2. Instruments

Several equipments for pesticides analysis were required including: fume cabinet, Quimis (Diadema, SP, Brazil); magnetic stirrer, Ikamag (Campinas, SP, Brazil); heating block, Sarge (Piracicaba, SP, Brazil). The determination of fenitrothion and deltamethrin were carried out by the high performance liquid chromatography (HPLC) equipment, Gilson (Middleton, WI, USA) equipped with a isocratic pump model 805, manual injector (20 µL loop) and with ultraviolet visible (UV) detection, 270 and 233 nm for fenitrothion and deltamethrin, respectively. The chromatographic column used for both pesticides were a C18 250x4.60 mm reversed-phase, Fusion-RP 80A 4µm particle size, Phenomenex (Madrid Avenue, Torrance, USA).

2.1.3. Samples

The wheat grains were collected from vertical silos in 2013 post-harvest, from the Brazilian Agricultural Research Corporation (Embrapa Wheat). Samples were received after cleaning and drying (up to a maximum of 60ºC) steps in the storage unit, packed in a polyethylene bag and stored at 4ºC at the Laboratory of Mycology, Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences, Federal University of Santa Catarina, Florianopolis, Santa Catarina.

2.2. Methods

2.2.1. Samples spiking pesticides before ozone treatment

Wheat grains were spiked using 100 µL of fenitrothion and deltamethrin standards solutions (1000 µg/mL), separately. The treated grains had a mc and a_w about to 12% & 20% and 0.6 & 0.9, respectively. In order to know the mc (2 g), the whole wheat grains were submitted to drying in an oven (105°C ± 5°C) up to constant weight through gravimetric method (AOAC, 2005). For a_w determination, wheat grains (2 g) were submitted to Aqua-Lab 4TE equipment. All analyses were performed in triplicate.

2.2.2. Ozone gas treatment in the storage laboratory pilots silos

The storage laboratory pilot silos with capacity of 25 x 10 cm (length x diameter) made by vinyl polychloride containing only two apertures: one for the input of O_3 gas and one for the output. The bottom part of the silos was filled with 400 g of wheat grains. The spiked grains were packed in the top part of the silos on the polyamide screen surface. The O_3 gas was applied to laboratory pilots silos, divided in Control Group (no O_3 gas) and Treated Group (60 µmol/mol) and were exposed of 30 up to 180 min. The O_3 gas generator system followed the procedures detailed by Giordano et al. (2012) with minor modifications, as described along this section: the compressed air pump was connected to an air impurities remover to filter the room air. The impurities removed were solid particles and humidity. Afterwards, the air filtered was conducted to the adjusted flow meter for 1 L/min and then the O_3 generator was calibrated to reach a concentration of 60 µmol/mol. The O_3 production (5 a 60 µmol/mol) was obtained through corona discharge process, in which an electrical discharge is oriented to a pure oxygen steam between the two electrodes. The O_3 gas produced was introduced through a tube into the input aperture of each chamber. The control chamber (without O_3 gas) was ventilated with “room air” in the same flow (1 L/min). The O_3 gas concentration measurement was performed by the iodinemetric test. For this, the O_3 concentration was measured in each chamber by the titration method on the outlet of the O_3 generator. The gas was bubbled into a potassium iodide solution (50 mL), acidified with 2.5 ml of sulfuric acid 1 N (pH below 2.0). The solution was titrated with sodium thiosulfate 0.005 N using a starch solution as indicator, according to APHA (1999).

2.2.3.. Fenitrothion analysis after ozone treatment

The used methodology was conducted according to D'Archivio et al. (2007). Each artificially contaminated wheat sample (5 g) was mixed with LC grade acetonitrile (60 mL) after the O_3 treatment. The mixture was stirred for 1 minute, followed by filtration. The filtrate (10 ml) was cleaned with a SPE Strata-X column in a flow rate of one drop per second. Prior to this step, the column was conditioned with dichloromethane (10 mL) followed by acetonitrile (10 mL) and Milli-Q water (10 mL). After the filtrate cleaning, the column was washed with 10 ml of LC grade water:methanol (95:5, v/v) and the pesticide slowly eluted with 1 ml of 100% LC grade acetonitrile and 1 mL of LC grade methanol. The eluate was evaporated to dryness using a heating block at 60 ºC with gentle nitrogen stream. The dry residue was then redissolved with 100 µL of mobile phase (acetonitrile:acidified water (H_3PO_4) 0.1%, 50:50, v/v). The extract (20 µL) was injected onto the LC/UV System. The mobile phase was delivered in a flow constant rate of 0.8 ml/min.

Fenitrothion quantification levels were performed by measuring the peak area at its retention time comparing to the standard solutions used for calibration curve (0.08-1 mg/L), with a correlation r = 0.991. Recovery was determined by spiking fenitrothion-free wheat samples with fenitrothion concentrations of 100 and 1000 mg/L in the same day and HPLC conditions.

2.2.4. Deltamethrin analysis after ozone treatment

The steps of extraction, cleaning and elution for deltamethrin were performed in the same way that fenitrothion analysis (D'Archivio et al., 2007). However, the dry residue was then redissolved with 100 µL of mobile phase (acetonitrile: water, 92:8, v/v). The extract (20 µL) was injected onto the LC/UV System. The mobile phase was delivered in a flow constant rate of 1 ml/min.

Deltamethrin quantification levels were performed by measuring the peak area at its retention time comparing to the standard solutions used for calibration curve (0.08-1 mg/L), with a correlation r = 0.992. Recovery was determined by spiking deltamethrin-free wheat samples with deltamethrin concentrations of 100 and 1000 mg/L in the same day and HPLC conditions.

2.2.5.Statistical Analysis

The obtained data were analyzed using analysis of variance (ANOVA) followed by Tukey or Bonferroni post-tests (when necessary). The data were expressed as mean ± standard deviation and the values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Effect of ozone treatment on fenitrothion residues degradation in wheat grains

The LC/UV method for fenitrothion chromatographic separation and the validation parameters (linearity, limit of detection - LOD, limit of quantification - LOQ, reproducibility, repeatability and recovery) showed to be quite adequate. Under the chromatographic conditions applied, the retention time (Rt) was of 14±0.5 min. Linearity was confirmed using the calibration curve for each fenitrothion concentration, which were linear at 0.08-1 mg/L, with a correlation factor equal to 0.991. The LOD and LOQ were of 0.01 and 0.2 mg/Kg, respectively. The mean recovery of the extraction method considering the concentrations equal to 100 and 1000 mg/L were 80% and 93%.

The residues of fenitrothion were significantly decreased after O_3 treatment after 180 min of exposure to 0.4 ± 0.2 mg/Kg (Control group: 1.2 ± 0.3 mg/Kg), which represents 69.4% of reduction (Fig. 2B). Several other treatment conditions in terms of time exposure and O_3 concentration were performed (data not shown), but showed inefficient. This active treatment was effective when the grains presented the highest humidity parameters (mc: 20% and a_w: 0.9). With mc: 12% e a_w: 0.6, however, there was not a significant difference between the Treated and Control group (Fig. 2A).

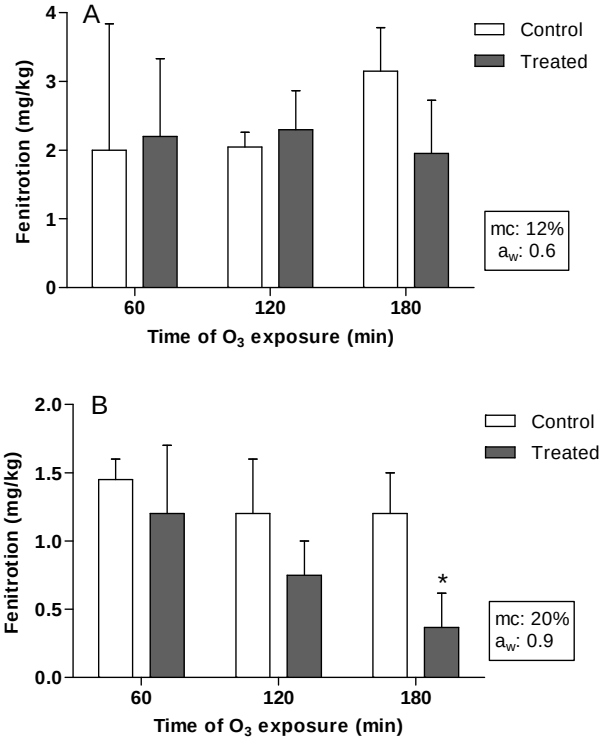

Fig. 2. Ozone gas effect on Fenitrothion at concentration of 60 µmol/mol and different exposure times (60, 120 and 180 min) and wheat grains humidity (A) moisture content: 12% and water activity: 0.6 and (B) moisture content: 20% and water activity: 0.9. *Statistically significant when compared to Control Group ($p<0.05$) by Bonferroni post-test.

3.2. Effect of ozone treatment on deltamethrin residues degradation in wheat grains

The LC/UV method for deltamethrin chromatographic separation and the validation parameters showed also to be quite adequate. The Rt was 6.0±0.5 min, considering a linear calibration curve in concentrations of 0.08-1 mg/L, with a correlation factor equal to 0.992. The LOD and LOQ were of 1.2 and 1.4 mg/Kg. The mean recovery of the extraction method

considering the concentrations to equal 100 and 1000 mg/L were 82 and 85%.

After 60 µmoL/moL O_3 treatment with a exposure time of 120 and 180 min, the deltamethrin residues were significantly reduced to 1.9 ± 0.9 mg/Kg and 1.5 ± 0.3 mg/Kg (Control: 9.8 ± 2.7 and 10.5 ± 1.3 mg/Kg), representing a reduction of 80.5% and 85.7% in the wheat grains that presented lower mc and a_w (12% and 0.6). However, no significant reductions were observed after 60 min of O_3 exposure (Fig. 3A). On the other hand, the grains with higher mc and a_w (20% and 0.9) presented the better results in relation to treatment in shorter time exposure, with a decreases in pesticide contents to 3.4 ± 1.8, 1.0 ± 0.6 and 1.5 ± 0.4 mg/Kg with 60, 120 and 180 min of O_3 exposure (Control: 10.45 ± 1.9, 8.8 ± 0.7 and 14.75 ± 0.2 mg/Kg), which represents a content reduction of 67.5, 88.0 and 89.8% (Fig. 3B).

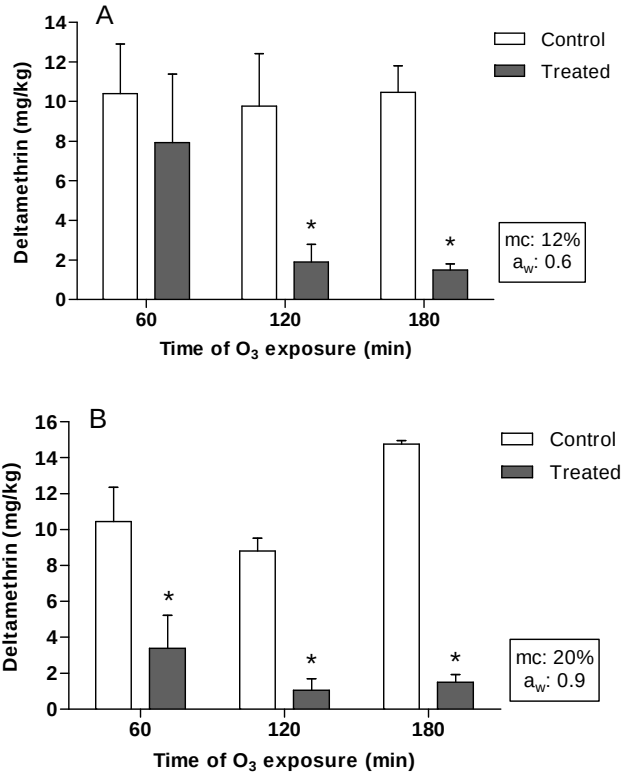

Fig. 3. Ozone gas effect on Deltamethrin at concentration of 60 µmol/mol and different exposure times (60, 120 and 180 min) and wheat grains humidity (A) moisture content: 12% and water activity: 0.6 and (B) moisture content: 20% and water activity: 0.9. *Statistically significant when compared to Control Group ($p<0.05$) by Bonferroni post-test.

4. Discussion

Pesticides residues may be persistent in wheat grains samples, even after the storage periods. Fenitrothion residues were detected in 30.8% of the imported wheat samples at levels of 0.032 to 0.445 mg/Kg, in the South Africa (Dalvie and London, 2009). In the U.S., 20.5% of the analyzed wheat samples were detected multiple pesticide residues. The most frequent were: chlorpyrifos, chlorpyrifos-methyl, cypermethrin, deltamethrin, malathion, pirimiphos-methyl, benomyl, carbendazim and thiophanate-methyl (CEC, 2005).

They also may remain on wheat sub-products, when are not totally eliminated during processing. Firmino et al. (2003) detected fenitrothion residues in 22.2% wheat flour samples, ranging from 0.07 to 0.40 mg/Kg. The same occurred with Caetano et al. (2005) that detected in 56.5% of the samples, ranging from 0.02 to 0.20 mg/Kg.

Balinova et al. (2007) showed that deltamethrin levels applied post-harvest on wheat was distinguished by low rate of degradation on the grain under practical storage conditions. One hundred and eighty days after treatment at an application rate of 0.5 mg/kg, the residues were between 0.03 and 0.2 mg/kg in the various types of flour. Two hundred and seventy days after treatment at a rate of 4 mg/kg, the residues in the flour were in the range of 0.4-1.5 mg/kg. In this study, the authors did not use any methodologies for pesticide degradation, the degradation occurred only during storage period, however, has not been effective enough to residues totally reduce.

In this sense, our study showed that the O_3 gas treatment was effective on fenitrothion and deltamethrin reduction at different exposure times (60, 120 and 180 min) and under different grain humidity (mc: 12% and a_w: 0.6 / mc: 20% and a_w: 0.9). The best results were found in the highest exposure times (120 and 180 min), which deltamethrin presented more

susceptibility to has its content reduced. In addition, the presence of water (in terms of humidity) in the grains may enhance the O_3 degradation effect, increasing the efficiency even under shorter exposure times.

Others studies showed the O_3 potential to pesticide degradation, especially in fruits and vegetables (Mendez et al., 2003; Rozado et al., 2008; Ikeura et al., 2011; Ikeura et al., 2013). Residual fenitrothion in vegetables (lettuce, cherry, tomatoes and strawberries) was efficiently removed using O_3 microbubbles (Ikeura et al., 2011). The same efficient results were observed by Ikeura et al. (2013), showing that O_3 microbubbles treatment had no effect on the colour and pulling strength of the leaves. These results indicate that the treatment by O_3 microbubbles is an effective method for removing the residues of fenitrothion in persimmon leaves and has relatively little effect on leaf quality characteristics. The expressive effect of O_3 in form of microbubbles agrees with our results regarding the cross related effect between humidity and pesticide degradation, where the presence of water positively contributes to de oxidizing effect of O_3.

Furthermore, O_3 exposure (10 µmol/mol, 60 min) was effective to degrade fenhexamid, cyprodinil and pyrimethanil fungicides residues from grapes (68.5, 75.4 e 83.7% of reduction, respectively) (Karaca et al., 2012). Kusvuran et al. (2012) also verified that aqueous O_3 (10 µmol/mol, 5 min) was enough to reduce pesticide residues, such as chlorothalonil, tetradifon and ethyl up to 92, 59.9 and 48.5% in lemon and 100, 56.6 and 40.4% in orange, respectively. Moreover, increasing of applied O_3 dosage was not significantly effective to improve the removal of the pesticides. This effect somehow agrees with our results in terms of exposure time (compare 120 vs. 180 min on Fig. 3A and 3B), since longer O_3 treatments did not improved the degradation efficiency.

However, according to the our knowledge, this should be the first work that has assessed the effect of O_3 on deltamethrin degradation in wheat grains. Moreover, previous studies showed that the application of O_3 gas in concentrations and exposure times used in this study (40 and 60 µmoL/moL, 30 to 180 min) have not affected quality attributes in the wheat grains, such as isolated starch oxidation and X-ray diffraction, lipid peroxidation, total protein profile and seed germination (Savi et al., 2014).

In addition to the monitoring of national programs for survey of pesticide residues in foods, alternatives to reduce these contaminants are important, since there is concern about the adverse effects of these compounds in the environment and human health. In this context, O_3 gas has a potential usefulness in the grains storage treatment to reduce the pesticides levels below of Brazilian regulation and thus ensure the food quality and security.

5. Conclusion

The effectiveness of O_3 gas on wheat grains artificially contaminated with fenitrothion and deltamethrin was observed and showed the best results when the grains present higher mc and a_w and where O_3 gas exposure occurs between 120 to 180 min. The presence of water enhances the O_3 degradation effectiveness and may be related to a contribution to the oxidizing effect of O_3.

The possibility of O_3 application in storage unit may be studied as effective technology for pesticides residues degradation commonly used in the wheat grains. This technology is a viable and effective alternative that can avoid the grain losses high due its advantages in chemical contaminants reduction.

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13 CONSIDERAÇÕES FINAIS

Para o conhecimento dos níveis de contaminação do trigo em grãos utilizados na pesquisa, foram avaliadas a umidade, atividade de água, presença de espécies fúngicas e micotoxina DON no trigo. Os grãos de trigo integral apresentaram níveis de umidade e atividade de água dentro dos limites esperados para trigo armazenado. Com relação aos fungos, importantes espécies fúngicas foram relatadas, incluindo as principais estudadas neste projeto (*F. graminearum*, *F. verticillioides*, *P. citrinum*, *A. flavus* e *A. parasiticus*). A presença de *F. graminearum* nos grãos de trigo podem indicar a contaminação por DON encontrado neste estudo, sendo que 47,2 % das amostras estavam contaminadas. Os níveis de contaminação apresentaram-se abaixo da legislação brasileira (2000 µg/kg) estabelecida neste ano (2013), com excessão de somente uma amostra (1,9%) que foi detectada acima do limite permitido. Considerando os níveis de DON que serão estabelecidos na legislação brasileira em 2017 (1000 µg/kg), 4 amostras (7,5%) analisadas estariam em desacordo com a legislação. Mesmo em baixas concentrações, a preocupação com as micotoxinas é constante, uma vez que o consumo de alimentos contaminados podem trazer prejuízos crônicos a saúde humana e animal. Por isso, o conhecimento da micoflora e os níveis de micotoxinas nos grãos podem contribuir para adoção de medidas de controle e estratégias de descontaminação para eliminar e/ou reduzir estes contaminantes.

Primeiramente, os compostos químicos foram testados como descontaminantes de fungos toxigênicos e micotoxinas encontradas no trigo em grãos. Os testes foram realizados *in vitro* para melhor entender o mecanismo de ação frente aos contaminantes e *in vivo* para avaliar a eficiência em grãos de trigo.

- Nanopartículas (NPs) de ouro foram testadas em cepas de fungos toxigênicos, sendo que nas concentrações utilizadas (0,05, 0,1 e 0,2 mg/L) não inibiram completamente o crescimento dos fungos. No entanto, foi possível verificar as modificações morfológicas das colônias e alteração na estrutura (hifas) dos fungos. Neste estudo, cepas de *F. verticillioides* e *A. flavus* apresentaram maior sensibilidade quando comparado com *P. citrinum*.

- Além das NPs de ouro, os compostos de zinco ($ZnSO_4$; $Zn(ClO_4)_2$; ZnO) também foram avaliados, incluindo as ZnO-NPs. Os compostos de zinco apresentaram resultados significativos, sendo que nos compostos de $ZnSO_4$ e $Zn(ClO_4)_2$ foram observados forte atividade anti-fúngica e anti-micotoxigênica, inibindo completamente o crescimento de *F. graminearum* e *P. citrinum*. Além disso, estes dois compostos foram capazes de inibir completamente a produção de DON por *F. graminearum*, sendo que somente o $Zn(ClO_4)_2$ foi capaz da inibição total da produção de AFB_1 e CTR por *A. flavus* e *P. citrinum*, respectivamente. A produção de DON por *F. graminearum* também foi inibida totalmente pela ação das ZnO-NPs. Em adição, alterações no metabolismo celular dos fungos foram observados na produção de conídios e formação das hifas, auxiliando na compreensão do mecanismo de ação destes compostos frente a estes contaminantes alimentares.
- Para *F. verticillioides* todos os compostos de zinco citados acima também foram estudados. Os compostos de $Zn(ClO_4)_2$ e ZnO-NPs foram os melhores com relação a inibição de crescimento fúngico, sendo que a produção de FBs (FB_1 e FB_2) foi completamente inibida com todos os compostos testados, exceto com o tratamento de ZnO em que o fungo produziu FB_2. Todos os compostos inibiram significativamente a produção de conídios e causaram alterações morfológicas (rupturas, deformações) nas hifas fúngicas. A mortalidade celular e produção de EROs demonstram forte potencial destes compostos nas alterações metabólicas do fungo, auxiliando na compreensão de seu mecanismo de ação.
- Nos estudo *in vivo*, os grupos tratados com $ZnSO_4$ and ZnO-NPs reduziram o crescimento de *F. graminearum* nas plantas de trigo, especialmente as ZnO-NPs que tiveram os melhores resultados em relação à atividade anti-fúngica. O mesmo ocorreu para a formação de DON, que foi reduzido após o tratamento, sendo que a micotoxina não foi detectada (<LOD) quando as plantas foram tratadas com ZnO-NPs. A presença de $ZnSO_4$ também reduziu significativamente a produção de DON pelo fungo. No entanto, as ZnO-NPs parecem ter melhores resultados devido ao maior contato com os grãos, servindo de proteção para inibição do *F. graminearum* inoculado artificialmente nas plantas de trigo durante período de floração.

Sendo assim, os grãos colhidos apresentaram baixa contagem e formação do DON quando comparado ao grupo controle. Como esperado, a MEV mostrou as características das hifas e conídios na superfície do grão artificialmente contaminado, entretanto, os tratamentos não apresentaram nenhum dano morfológico observado nos grãos de trigo. Além disso, mesmo após aplicação de zinco nas plantas, os níveis deste composto nos grãos colhidos estavam dentro dos níveis recomendados internacionalmente para consumo diário.

Em segundo momento, o O_3 gasoso foi utilizado como método químico de descontaminação. Os testes foram realizados *in vitro* e *in vivo* (trigo em grãos) para avaliação de seus efeitos frente aos contaminantes.

- Nas pesquisas *in vitro*, a eficiência do gás O_3 foi testada para avaliar o mecanismo de ação deste descontaminante frente aos principais fungos toxigênicos contaminantes do trigo. Entre as espécies testadas, *F. graminearum* e *P. citrinum* foram os mais sensíveis ao tratamento na concentração de gás O_3 de 60 µmol/mol a 120 min de exposição, sendo inibidos completamente. Já *F. verticillioides*, *A. flavus* e *A. parasiticus* foram mais resistentes ao tratamento, no entanto, houve significativa diminuição do crescimento no maior tempo de exposição (120 min). Com relação a germinação dos conídios, *F. graminearum* e *P. citrinum* foram os mais sensíveis, no entanto, a germinação de todos os fungos testados foi fortemente inibida pelo tratamento com gás O_3 (60 µmol/mol por 120 min). Em adição, foram observados alterações morfológicas na estrutura de todos os fungos. *F. graminearum* foi o fungo que apresentou maior sensibilidade a mortalidade das hifas. Além disso, foram observados a produção de EROs nas hifas fúngicas, o que já poderia ser esperado devido a forte ação oxidante do gás O_3.
- Nas pesquisas *in vivo*, o gás O_3 foi avaliado frente ao principal fungo de campo (*F. graminearum*) contaminante do trigo em grão e a micotoxina DON. Em adição, os efeitos do tratamento sobre parâmetros físicos e bioquímicos dos grãos também foram analisados. Neste estudo, a contaminação das amostras foi realizada artificialmente para ter real certeza do efeito descontaminante do gás O_3. A concentração de 40 e 60 µmol/mol foram eficientes em inibir o crescimento do fungo nos grãos de trigo, principalmente no tempo de exposição de 180 min,

o qual foi inibido completamente por ambas concentrações. Com relação a contaminação por DON, os níveis de toxina foram reduzidas completamente por 120 min de exposição na concentração testada (60 µmol/mol). Como esperado, os níveis de DON no pericarpo foram reduzidos com maior facilidade quando comparado com a contaminação no endosperma do grão. A concentração e o tempo de exposição do gás O_3 que demonstrou melhor eficiência frentes aos contaminantes, foram escolhidos para as análises dos parâmetros de qualidade do grão após o tratamento. Devido ao alto conteúdo de amido nos grãos de trigo (80%), tanto a oxidação (carbonil e carboxil), quanto DRX foram avaliados. Neste caso, somente o conteúdo de carboxil foi significativamente maior quando comparado com o amido Controle. Não houve diferença entre amido isolado do grão Controle e Tratado nos testes de carbonil e DRX. Em adição, nenhuma alteração ocasionada pelo tratamento com gás O_3 foi verificado quanto aos parâmetros analisados de peroxidação lipídica e análise de proteínas. No MEV foram verificadas as características da microestrutura do grão Controle e Tratado, assim como o amido isolado. Entretanto, nenhuma alteração visível da morfologia do grão e do amido foi observada. Por fim, no teste de germinação das sementes Controle e Tratadas, somente o tratamento com gás O_3 por 180 min de exposição demonstrou diminuir significativamente a germinação. No entanto, nenhuma alteração no crescimento dos coleóptilos e raiz seminal das sementes foi observada. Este estudo mostra que o gás O_3 pode ser efetivo na inibição de *F. graminearum* e DON em grãos de trigo, principalmente na concentração de 60 µmol/mol por 120 min de exposição, a qual não alterou nenhuma das características físicas e bioquímicas analisadas no trigo.

- O gás O_3 também foi testado frente aos fungos de armazenamento, tais como: *A. flavus* e *P. citrinum*, que frequentemente são encontrados nos grãos de trigo, responsáveis pela formação de AFLs e CTR. Na maior concentração de O_3 (60µmoL/moL), *A. flavus* and *P. citrinum* foram completamente inibidos após 180 min de exposição. Em adição, a mesma exposição de O_3 reduziu os níveis de AFB_1 e AFB_2 (12,51 e 41,06 µg/Kg) significativamente quando comparado ao grupo Controle (231,88 e 265,79 µg/Kg). Além disso, os níveis de CTR também foram reduzidos nas duas concentrações pesquisadas (40 e 60µmoL/moL) após 180 min de exposição (103,64 e 42,90

µg/Kg) quando comparado ao grupo Controle (146,85 e 173,51 µg/Kg).

- Por fim, o gás O_3 foi avaliado quanto a sua eficiência em reduzir resíduos de agrotóxicos, especialmente os inseticidas (fenitrotiona e deltametrina) que são aplicados sobre os grãos de trigo nas correias transportadoras na etapa anterior ao armazenamento nos silos. O inseticida fenitrotiona foi reduzido (69,4%) após o tratamento com gás O_3 em 180 min de exposição quando os grãos apresentaram umidade e atividade de água de 20% e 0,9, respectivamente. Quando os grãos apresentam menor umidade e atividade de água (12% e 0,6), não houve diferença significativa entre os grupos Controle e Tratado. Por outro lado, após aplicação de gás O_3 na concentração de 60 µmoL/moL e maiores tempos de exposição (120 e 180 min), a deltametrina foi significativamente reduzida em ambos os valores de umidade e atividade de água, sendo que os grãos com maior umidade e atividade de água apresentaram os melhores resultados nos diferentes tempos de exposição (60, 120 e 180 min - redução de 67,5, 88,0 e 89,8%, respectivamente).

Diante dos resultados encontrados neste trabalho podemos verificar que novas estratégias de controle e descontaminação com os compostos químicos, especialmente os de zinco, poderiam aumentar a eficiência frente ao crescimento de fungos e formação das micotoxinas. Especialmente frente a *F. graminearum* e DON que são contaminantes das plantas de trigo e frequentemente encontradas nos grãos de trigo colhidos.

Além disso, a descontaminação com gás O_3 demonstrou ser um método químico promissor a ser aplicado nas indústrias e unidades armazenadoras durante o período de armazenamento dos grãos de trigo, a fim de reduzir a contaminação e garantir a segurança alimentar do consumidor, especialmente por ter vantagens de ser internacionalmente reconhecido como seguro e não deixar resíduos nos alimentos.

Quanto à viabilidade prática do uso dos descontaminantes químicos, estes podem ter aplicações diretas nos alimentos quando em doses adequadas e aprovadas por órgãos nacionais e internacionais (FAO, FDA, WHO).

Entre os compostos utilizados neste estudo, podemos citar o sulfato de zinco e o óxido de zinco, que além de já ser utilizado como suplemento alimentar com aprovação pela FAO e FDA, apresentaram forte atividade anti-fúngica e anti-

micotoxigênica. Em quantidades adequadas esses poderiam ser aplicados no campo para controlar o crescimento de fungos nos grãos junto ou separadamente dos compostos químicos de controle atualmente utilizados.

Além disso, os resíduos destes compostos químicos poderiam atribuir a fortificação ao alimento, já que especialmente no trigo, o processamento reduz substancialmente os níveis de zinco. A aplicação destes compostos no campo, poderia fornecer conhecimento da melhor concentração utilizada para atingir ambas as vantagens, sua atividade anti-fúngica e anti-micotoxigênica e também a fortificação dos grãos, o que irá agregar valor ao produto e manter a qualidade dos grãos para o consumidor.

Por outro lado, na armazenagem dos grãos, as altas perdas na produção e qualidade sempre foram problemas para os produtores no Brasil e em todo o mundo. Tecnologias como o gás O_3 já vem sendo utilizadas na armazenagem dos grãos em países como os Estados Unidos para evitar a infestação de insetos (GUZEL-SEYDIM; GREENE; SEYDIM, 2004; USA, 1997). No Brasil, essa tecnologia é utilizada especialmente em vegetais, frutas, embalagens e para desinfecção de água. Atualmente, os controles mais utilizados para evitar as pragas e micro-organismos nos grãos são os fungicidas e inseticidas protetores, no entanto, o problema está na resistência. Por isso, a busca de novas alternativas como o O_3 é constante. Além de reduzir pragas como os insetos, e o crescimento de bactérias, nosso estudo demonstrou que o O_3 tem eficiência sobre a redução de fungos, degradação de micotoxinas e agrotóxicos. E as vantagens de não deixar resíduos nos alimentos e ser rapidamente difundido em O_2, tornam o O_3 ainda mais atrativo para a indústria de alimentos. Além disso, nossos resultados com grãos de trigo mostraram que em concentrações efetivas (contra fungos, micotoxinas e agrotóxicos), o tratamento com O_3 não é capaz de causar alterações físico-bioquímicas nos grãos de trigo armazenados.

Atualmente a estrutura das unidades armazenadores no Brasil pode ser um problema quanto a aplicação desta tecnologia nos silos de armazenamento, no entanto, o seu uso na indústria está crescendo e atingindo outros setores, como nos moinhos e máquinas embaladoras. E com estudos que mostram rentabilidade do seu uso em grãos armazenados (PEREIRA et al., 2008), sua eficiente distribuição utilizando sistemas de aeração nos silos

(ROZADO, 2013) e sua eficácia frente aos principais contaminantes dos grãos estudados, esta tecnologia poderá cada vez mais, despertar a atenção dos produtores de grãos para adquirir uma estrutura suficiente e adequada para o uso do gás O_3. Esta tecnologia é uma alternativa viável e eficaz que pode evitar as altas perdas de grãos e manter a qualidade do alimento para o consumidores.

14 SUGESTÕES PARA PESQUISAS FUTURAS

Como sugestões para pesquisas futuras, é possível citar algumas proposições que abrangem o tema proposto neste estudo:

- Aplicação dos compostos de zinco juntamente com os agrotóxicos em plantações de trigo em larga escala, procurando avaliar efeito sinérgico.

- Estudar o efeito do gás O_3 na redução de micro-organismos naturalmente presentes no trigo em grãos.

- Analisar os subprodutos de degradação por gás O_3 das micotoxinas analisadas, tais como, DON, AFLs e CTR. Assim como, os subprodutos de agrotóxicos, fenitrotiona e deltametrina.

- Avaliar a viabilidade econômica para aplicação em larga escala de gás O_3 nos silos de armazenamento juntamente com sistemas de ventilação.

- Propor implementação de estruturas e equipamentos necessários para a aplicação de gás O_3 em silos de armazenamento de trigo em grãos no Brasil.

ANEXOS

ANEXO A - Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil

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Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil

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ANEXO B - Biological Activity of Gold Nanoparticles towards Filamentous Pathogenic Fungi

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Biological Activity of Gold Nanoparticles towards Filamentous Pathogenic Fungi

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ANEXO C - Antifungal properties of Zinc-compounds against toxigenic fungi and mycotoxin

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Original article

Antifungal properties of Zinc-compounds against toxigenic fungi and mycotoxin

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ANEXO D - Effect of zinc compounds on Fusarium verticillioides growth, hyphae alterations, conidia, and fumonisin production

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Geovana D Savi,[a] Vinícius Vitorino,[a] Adailton J Bortoluzzi[b] and Vildes M Scussel[a]*

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ANEXO E - Effects of Ozone Gas Exposure on Toxigenic Fungi Species from *Fusarium*, *Aspergillus*, and *Penicillium* Genera

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ANEXO F - Ozone treatment efficiency on *Fusarium graminearum* and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum* L.) quality and germination

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ANEXO G - Ozone treatment efficiency in *Aspergillus* and *Penicillium* growth inhibition and mycotoxin degradation of stored wheat grains (*Triticum aestivum* L.)

Ozone Treatment Efficiency in *Aspergillus* and *Penicillium* Growth Inhibition and Mycotoxin Degradation of Stored Wheat Grains (*Triticum aestivum* L.)

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